B cell- and antibody-mediated protection in lymphotoxin β receptor-deficient mice during *Toxoplasma gondii* infection

Inaugural dissertation

for the attainment of the title of doctor
in the Faculty of Mathematics and Natural Sciences
at the Heinrich Heine University Düsseldorf

presented by

Marcel Helle

from Brilon

Düsseldorf, February 2025

from the Institute of Medical Microbiology and Hospital Hygiene
at the Heinrich Heine University Düsseldorf

Published by permission of the
Faculty of Mathematics and Natural Sciences at
Heinrich Heine University Düsseldorf

Supervisor: Univ.-Prof. Dr. Klaus Pfeffer

Co-supervisor: Univ.-Prof. Dr. Johannes Hegemann

Date of oral examination: July 10, 2025

Abstract

The lymphotoxin β receptor (LTβR) is essential for the development and maintenance of secondary lymphoid organs and the induction of effective immune responses against various pathogens, including the obligate intracellular parasite *Toxoplasma gondii*.

T. gondii causes toxoplasmosis which can lead to severe complications in immunocompromised individuals and fetuses. While toxoplasmosis may be asymptomatic or cause mild flu-like symptoms in immunocompetent hosts, the parasite can persist in the body for life, with a potential risk of reactivation. With no vaccine approved for human use and approximately 30 % of the global population infected, *T. gondii* poses a significant public health concern.

LTβR-deficient (LTβR$^{-/-}$) mice exhibit profound impairments in both innate and adaptive immunity, resulting in reduced survival compared to wild-type (WT) counterparts. While previous studies have shown that the generation of *T. gondii*-specific, class-switched antibodies is impaired in LTβR$^{-/-}$ mice, the role of antibody-mediated protection and the state of the B cell compartment outside the spleen and lymph nodes in these mice had not been fully explored. Therefore, these topics were investigated by examining the B cell compartment in the bone marrow (BM), peritoneal cavity (PerC), and blood of LTβR$^{-/-}$ mice, and by implementing a passive immunization protocol, where serum from *T. gondii*-infected WT mice was transferred to recipient mice.

LTβR$^{-/-}$ mice exhibited distinct alterations in multiple B cell subpopulations compared to WT counterparts: mature B cell frequencies were elevated in the BM and remained, in contrast to those of WT mice, unaffected by infection. Plasma cells of LTβR$^{-/-}$ mice expressed predominantly immunoglobulin M (IgM) rather than IgA. Additionally, the T cell compartment in the LTβR$^{-/-}$ BM was affected, exhibiting reduced double-negative (DN) T cell and increased CD4^{+} and CD8^{+} T cell frequencies. In the PerC, B-2 and B-1b – but not B-1a – B cell frequencies were also increased compared to WT counterparts. On day 9 p.i., the LTβR$^{-/-}$ PerC was dominated by neutrophils rather than T cells, potentially exacerbating immunopathology in these mice.

Furthermore, the passive immunization produced ambiguous results regarding protection against *T. gondii* in LTβR$^{-/-}$ mice and, additionally, led to a partial suppression of the endogenous *T. gondii*-specific antibody production in WT recipients; this effect might be caused by inhibitory effects of transferred high-affinity IgG antibodies.

In summary, this dissertation provides new insights into diverse immune cell alterations, particularly in the B cell compartment, in the absence of LTβR signaling during protozoan parasite infection, paving the way for further research and potential therapeutic strategies targeting both the LTβR and *T. gondii.*

Zusammenfassung

Der Lymphotoxin-β-Rezeptor (LTβR) ist essenziell für die Entwicklung und den Erhalt sekundärer lymphoider Organe sowie für die Induktion effektiver Immunreaktionen gegen verschiedenste Pathogene, einschließlich des obligat-intrazellulären Parasiten *Toxoplasma gondii*. *T. gondii* verursacht Toxoplasmose, eine Krankheit, die bei immungeschwächten Personen und Föten zu schweren gesundheitlichen Problemen führen kann. Zwar ruft Toxoplasmose bei immunkompetenten Wirten meist nur milde grippeähnliche Symptome hervor, jedoch kann der Parasit auch in diesen Fällen lebenslang im Körper überdauern, wodurch das Risiko einer Reaktivierung besteht. Da es derzeit keinen für den Menschen zugelassenen Impfstoff gibt und etwa 30 % der Weltbevölkerung infiziert sind, stellt *T. gondii* ein erhebliches Problem für die öffentliche Gesundheit dar.

LTβR-defiziente (LTβR$^{-/-}$) Mäuse weisen Beeinträchtigungen sowohl der angeborenen als auch der adaptiven Immunität auf, was nach einer Infektion zu einer geringeren Überlebensrate im Vergleich zu Wildtyp-Mäusen (WT) führt. Frühere Studien zeigten, dass die Bildung *T. gondii*-spezifischer Antikörper in LTβR$^{-/-}$-Mäusen beeinträchtigt ist; allerdings sind das Ausmaß des schützenden Effekts von Antikörpern und der Zustand des B-Zell-Kompartiments außerhalb von Milz und Lymphknoten in diesen Mäusen nur unzureichend erforscht. Daher wurde in dieser Dissertation das B-Zell-Kompartiment im Knochenmark, in der Peritonealhöhle und im Blut von LTβR$^{-/-}$-Mäusen sowie der Antikörper-vermittelte Schutz gegen *T. gondii* mittels passiver Immunisierungen untersucht.

Die LTβR$^{-/-}$-Mäuse wiesen verschiedene Veränderungen in den B-Zell-Subpopulationen im Vergleich zu WT-Mäusen auf: Die Frequenzen reifer B-Zellen im Knochenmark waren erhöht und blieben von der Infektion unbeeinflusst, im Gegensatz zu denen der WT-Mäuse. LTβR$^{-/-}$-Plasmazellen exprimierten größtenteils Immunoglobulin M (IgM); IgA Plasmazellen waren kaum nachweisbar. Auch die T-Zell-Subpopulationen im LTβR$^{-/-}$-Knochenmark waren verändert, da die Frequenzen an doppelt-negativen (DN) T-Zellen reduziert und an CD4^{+} und CD8^{+} T-Zellen erhöht waren. Außerdem waren in der LTβR$^{-/-}$-Peritonealhöhle die Frequenzen der B-2- und B-1b-Zellen – nicht jedoch der B-1a-Zellen – im Vergleich zu WT-Mäuse erhöht. An Tag 9 der Infektion wurde die LTβR$^{-/-}$-Peritonealhöhle von Neutrophilen statt von T-Zellen dominiert, was möglicherweise vorhandene immunpathologische Effekte in diesen Mäusen verschlimmerte.

Während die passiven Immunisierungen zu keinen eindeutigen Ergebnissen hinsichtlich des vermittelten Schutzes gegen *T. gondii* in LTβR$^{-/-}$-Mäusen führte, wurde in WT-Empfängern teilweise die endogene Produktion *T. gondii*-spezifischer Antikörper unterdrückt; möglicherweise geschah dies aufgrund der transferierten hochaffinen IgG-Antikörper, welche inhibierend auf die endogene Antikörperproduktion wirken können.

Zusammengefasst liefert diese Dissertation neue Einblicke in die Veränderungen verschiedener Immunzellpopulationen, insbesondere im B-Zell-Kompartiment, bei nicht-infizierten sowie *T. gondii*-infizierten LTβR$^{-/-}$-Mäusen. Diese Erkenntnisse ebnen den Weg für weitere Forschung und mögliche therapeutische Strategien, die sowohl auf den LTβR als auch auf *T. gondii* abzielen.

Table of contents

Abstract I
Zusammenfassung II
1. Introduction 1
1.1 The immune system of mice and humans – a brief overview 1
1.1.1 Hematopoiesis in the adult bone marrow 3
1.1.2 B cell development and subpopulations 4
1.1.3 Activation of mature B cells and the generation of high-affinity antibodies in germinal centers 8
1.2 Toxoplasma gondii 10
1.2.1 Life cycle and routes of infection 10
1.3 Innate immunity against *T. gondii* 12
1.3.1 The Interferon family 14
1.3.2 IFNγ-induced cell-autonomous immunity against *T. gondii* 15
1.3.3 *T. gondii* counter measures against innate immune responses 15
1.4 The lymphotoxin β receptor (LTβR) and the lymphotoxin signaling network 16
1.4.1 The canonical and alternative NF-κB signaling pathways 19
1.4.2 LTβR-deficient (LTβR$^{-/-}$) mice 20
1.4.3 LTβR$^{-/-}$ mice during infection 21
2. Aim of the thesis 23
3. Publications 24
3.1 Lymphotoxin Beta Receptor$^{-/-}$ Mice Display Altered B and T Cell Subpopulations in the Bone Marrow and Peritoneal Cavity after *Toxoplasma gondii* Infection 25
3.2 Antibody-mediated Protection in Lymphotoxin β Receptor-deficient Mice Against *Toxoplasma gondii* 85
3.3 Lymphotoxin β Receptor: a Crucial Role in Innate and Adaptive Immune Responses Against *Toxoplasma gondii* 126
4. Discussion and concluding remarks 154
5. References 160
Appendix 184
Figures 184
Abbreviations 184
Statutory Declaration / Eidesstattliche Erklärung V
Acknowledgments VI

1. Introduction

1.1 The immune system of mice and humans – a brief overview

The vertebrate immune system is a complex network responsible for the defense against harmful pathogens, including bacteria, viruses, fungi and parasites, while simultaneously allowing coexistence with beneficial microorganisms, such as the diverse gut microbiome. This finely tuned, evolutionary adapted system is vital for maintaining health, and any malfunction can have serious consequences for the organism it safeguards. The immune system is typically divided into innate and adaptive responses, but these two components are closely interconnected and influence each other in a bidirectional manner. Innate immunity acts as the first line of defense against invading pathogens [1]. This includes anatomical and chemical barriers, such as the skin and broad-spectrum antimicrobial proteins, which prevent pathogen invasion. If invading pathogens overcome these defenses, innate immune effectors respond rapidly, while components of the adaptive immune response require more time and partially rely on the innate response for activation. However, before a pathogen can be attacked, it must first be detected. Pathogen derived molecules, known as pathogen-associated molecular patterns (PAMPs), are recognized by pathogen-recognition receptors (PRRs), which are expressed by many cells but are particularly abundant in sensor cells such as dendritic cells (DCs) and macrophages. Notably, the ability of these receptors to recognize conserved molecular patterns is encoded in the genome, whereas the antigen receptors of adaptive immune cells are generated through somatic recombination and can recognize a broader array of antigens, including those not pre-encoded in the genome [1,2].

Both innate and adaptive immune responses rely on immune cell populations with distinct characteristics and functions. While some immune cells are primarily associated with either innate or adaptive immunity, others serve as a bridge between these systems. Immune cells are classified as myeloid or lymphoid based on their origin (see subsection 1.1.1.). The myeloid lineage includes granulocytes (neutrophils, basophils, eosinophils), monocytes, macrophages, and most DC populations, and is primarily associated with innate immunity. In contrast, the lymphoid lineage includes B cells and T cells, which are key players in the adaptive immune response, as well as natural killer (NK) cells, innate lymphoid cells (ILCs) and plasmacytoid DCs (pDCs) [3,4]. Pathogen recognition triggers DCs and macrophages to produce proinflammatory molecules known as cytokines, which subsequently activate other immune cells, initiating a proinflammatory cascade. Activated immune cells deploy a range of mechanisms to combat infection, including the release of cytotoxic granules by granulocytes and NK cells to destroy infected cells, the induction of phagocytosis by macrophages to engulf and eliminate pathogens, the promotion of controlled cell death (apoptosis) to remove infected or damaged cells, and the recruitment of additional immune cells to the site of infection to amplify the immune response [1]. Antigen-presenting cells (APCs), such as DCs and macrophages, play an important role in linking innate

and adaptive immune response. They process pathogen-derived antigens and present them *via* major histocompatibility complex (MHC) molecules to the T cell receptors (TCR) of naïve T cells, which is essential for their activation and differentiation into various functional subsets.

T cells are broadly categorized based on their expression of CD4 or CD8 co-receptors, which define two heterogeneous groups with distinct phenotypes and functions [5]. $CD8^+$ T cells are activated through TCR-recognition of peptides presented by MHC class I (MHCI) molecules, accompanied by co-stimulatory signals. This triggers the release of cytokines and cytotoxic granules containing effector proteins, such as perforin and granzyme, which induce apoptosis of target cell [1,6,7]. In contrast, $CD4^+$ T cells recognize antigens presented by MHCII molecules and, depending on received cytokine stimuli, differentiate into specialized subpopulations, including T helper 1 cells (Th1), Th2, Th9, Th17, Th22, follicular helper T cells (T_{FHs}), regulatory T cells (T_{regs}) and $CD4^+$ cytotoxic T lymphocytes (CTLs) [5]. Each subset has a unique cytokine profile and functional role that can also affect other immune cell populations. For example, T_{FH} cells collaborate closely with B cells to facilitate survival, proliferation, plasma cell (PC) differentiation, somatic hypermutation, class-switch recombination, and chemotactic responses [8]. Additionally, natural killer T (NKT) cells possess both NK cell and T cell characteristics, bridging innate and adaptive immunity. Unlike conventional T cells, NKT cells predominantly lack CD4 and CD8 co-receptors but express invariant TCRs and NK cell receptors and recognize microbial lipids presented by CD1 molecules rather than peptide-MHC complexes [1].

In addition to cellular immunity, the immune system also employs humoral mechanisms, including antibodies, complement proteins, and other soluble factors in bodily fluids, to neutralize and eliminate pathogens [1]. Naïve mature B cells are classified as conventional (or 'B-2') cells, which includes both follicular (FO) and marginal zone (MZ) B cells, and B-1 cells (see subsection 1.1.2). After antigen (Ag) encounter, B cells develop into plasmablasts and plasma cells, which are the main producers of antigen-specific antibodies (also named 'adaptive antibodies'; [9]) in the adaptive immune response. Conversely, B-1 cells are a major source of natural antibodies (NAbs), which are secreted spontaneously without prior antigen exposure and are therefore part of the innate "first line of defense" against invading pathogens. Additional functions of NAbs include assistance in B cell receptor (BCR) repertoire selection, regulation of B cells responses, and clearance of apoptotic debris [10]. Furthermore, the small population of regulatory B cells (B_{regs}) helps to maintain immune homeostasis and prevent excessive inflammation by secreting interleukin-10 (IL-10), in a role similar to that of T_{regs} [11].

In general, a hallmark of adaptive immunity is its capacity to generate immunological memory, which enables faster and more robust responses upon re-exposure to previously encountered pathogens. This is mediated by long-lived, antigen-specific memory B and T cells, which are generated during the primary immune response and can persist for years, decades, or even a lifetime. Importantly, immunological

memory forms the foundation of vaccination, where the immune system is 'trained' with non-harmful antigens to enhance protection against future infections with the targeted pathogen [1].

1.1.1 Hematopoiesis in the adult bone marrow

A schematic overview of immune cell hematopoiesis is presented in Figure 1. While this model provides a framework for this dissertation, it is important to note that the process is more complex and continues to be refined through ongoing research [3]. In general, immune cell development in the adult bone marrow (BM) begins with hematopoietic stem cells (HSCs), which are rare, self-renewing cells capable of giving rise to all blood lineages through a stepwise differentiation process [3]. HSCs differentiate into multipotent progenitor cells (MPPs), which retain the ability to differentiate into various cell types but lose their self-renewal capacity. Then, according to the classic CMP/CLP model, MPPs can differentiate into either common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs), marking a major bifurcation in the hematopoietic hierarchy. Consequently, terminally differentiated immune cells are typically classified as either myeloid or lymphoid, depending on their progenitor origins. However, more recent research has identified lymphoid-primed multipotent progenitor cells (LMPPs) as an alternative commitment step, which arise from MPPs and have both lymphoid as well as limited myeloid (granulocyte-macrophage) lineage potential [12-14], as shown in Figure 1. CMPs further differentiate into megakaryocyte-erythrocyte progenitors (MEPs) or granulocyte-macrophage progenitors (GMPs). While LMPPs also have the potential to generate GMPs, they are primarily biased toward the lymphoid lineage, predominantly giving rise to CLPs. CMP-derived MEPs can produce erythrocytes or, through further differentiation into megakaryocytes, produce platelets. GMPs, on the other hand, can differentiate into a variety of myeloid cells, including DCs, granulocytes (neutrophils, eosinophils and basophils), or monocytes, which can further develop into macrophages or monocyte-derived dendritic cells (moDCs). CLPs give rise to terminally differentiated lymphoid cells, including T cells, B cells, NK cells, ILCs and pDCs [3]. DCs are further classified into conventional DCs (cDCs), which perform general DC-associated functions such as antigen presentation, co-stimulation and cytokine secretion, and pDCs, which are dedicated to the secretion of large amounts of type I interferons IFNs (see subsection 1.3.1) [15]. The development and plasticity of these different DC subsets is complex and not yet fully understood [16,17].

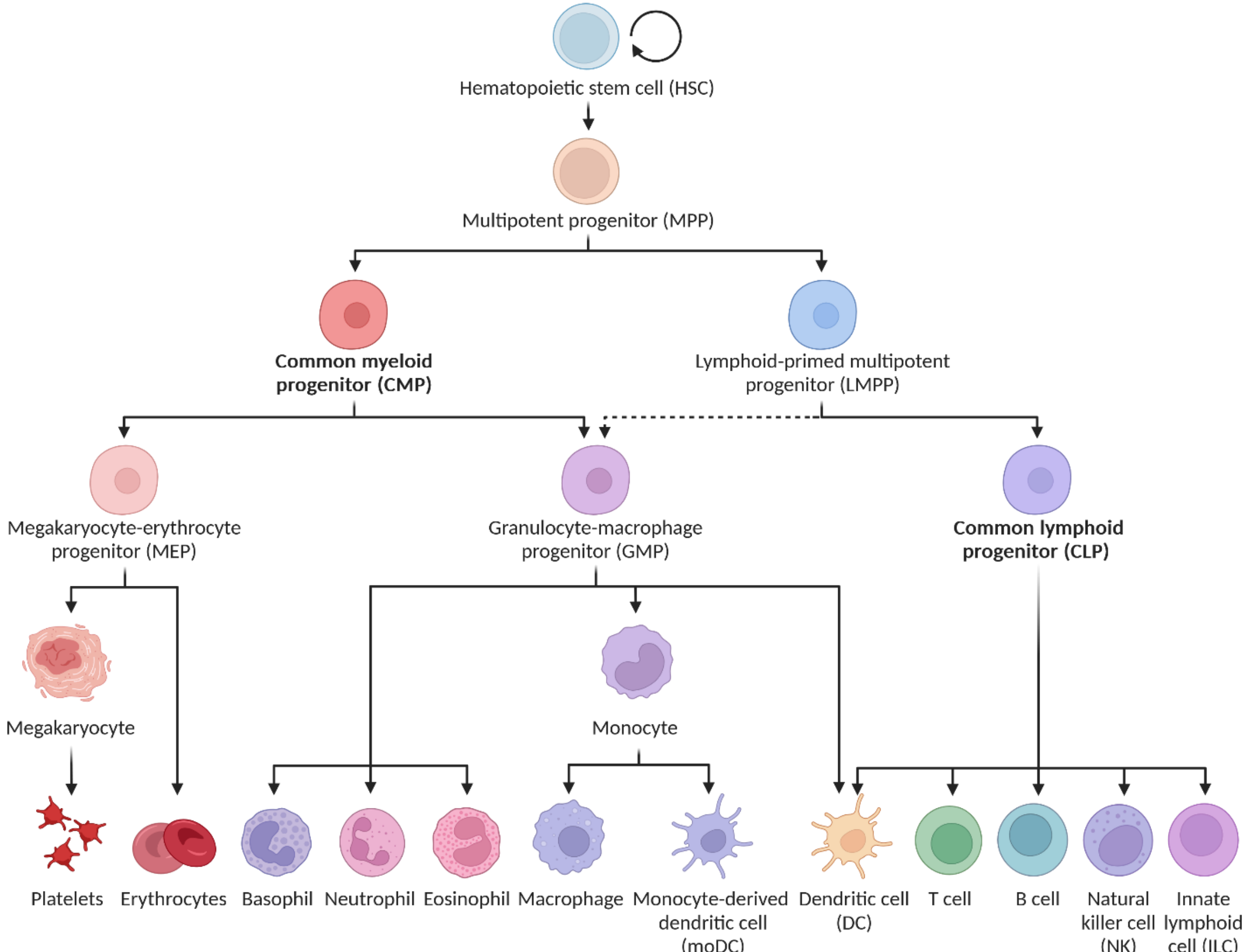


Figure 1: Hematopoiesis in the adult bone marrow. The differentiation of hematopoietic stem cells (HSCs), which have self-renewal capacities, into terminally differentiated immune cells is a stepwise process. Early in development, HSCs give rise to multipotent progenitors (MPPs), which then diverge into either common myeloid progenitors (CMPs) or lymphoid-primed multipotent progenitors (LMPPs). From there, CMPs further differentiate into megakaryocyte-erythrocyte progenitors (MEPs) or granulocyte-macrophage progenitors (GMPs), and both are part of the myeloid branch. Although LMPPs have the potential to also produce GMPs, they are biased toward the lymphoid lineage, giving rise to common lymphoid progenitors (CLPs). These CLPs can terminally differentiate into T cells, B cells, natural killer (NK) cells, innate lymphoid cells (ILCs) and plasmacytoid DC (pDCs). Continuing the myeloid branch, CMP-derived megakaryocyte-erythrocyte progenitors (MEPs) differentiate into erythrocytes or, after further maturation into megakaryocytes, produce platelets. CMP- and LMPP-derived GMPs can give rise to various immune cells: granulocytes (basophils, neutrophils, and eosinophils), dendritic cells (DCs) and, through differentiation into monocytes, macrophages and monocyte-derived DCs (moDCs). Created with BioRender.com.

1.1.2 B cell development and subpopulations

B cells can be divided into two subpopulations: B-1 and B-2 cells. B-1 cells are primarily found in body cavities, such as the peritoneal and pleural cavities, and exhibit more innate-like characteristics compared to B-2 cells. B-2 cells, also known as conventional B cells, perform classic functions as part of the adaptive immune response and are located in the BM, spleen, secondary lymphoid organs (SLOs) and peripheral tissues [1].

B-2 cells: Most B-2 cells, except for long-lived memory B cells and PCs, require continuous replenishment in adult mice and humans. B cell development begins in the BM with HSCs, which differentiate through a stepwise process into CLPs. Throughout development, B cells reside in specialized niches within the BM, where they receive critical signals from surrounding non-lymphatic stromal cells, including cytokines for gene expression and chemokines for proper spatial localization [18]. A primary goal of B cell development is the sequential assembly, expression, and signaling of a functional B cell receptor (BCR), a cell surface immunoglobulin (Ig) receptor that recognizes specific antigenic epitopes. Structurally, the BCR is a membrane-bound antibody composed of two rearranged Ig heavy (IgH) and Ig light (IgL) chains, but with the addition of an anchoring cytoplasmic tail. Unlike typical innate PRRs, the specificity of the BCR (and antibody) is not hardcoded into the genome. Instead, rearrangement of multiple genomic loci allows for more diversity, which is further enhanced through the processes of somatic hypermutation and class-switching of the IgH chain following after antigen encounter [1].

Summarized, the stages of B cell development from CLPs in the BM are classified as pro- (progenitor), pre- (precursor), immature- and transitional B cells (Fig. 2), and they can be identified by specific surface markers and intracellular transcription factors [19,20]. During the pro-B cell stage, IgH chain (IgH) gene rearrangement is mediated by the recombination-activating genes 1 and 2 (RAG1 and RAG2), which are essential for the recombination of variable (V), diversity (D), and joining (J) segments of the IgH chain gene locus (V(D)J recombination) [21,22]. In pre-B cells, the functionality of the rearranged IgH is tested by expression of a pre-BCR complex, which consists of the IgH paired with surrogate IgL chains (VpreB and λ5), along with the signaling molecules Igα (CD79a) and Igβ (CD79b) [23-26]. A functional pre-BCR acts as a checkpoint during B cell development, with positive selection allowing pre-B cells to survive, to proliferate, and to initiate IgL chain rearrangements (V and J segments) [27-29]. Once both IgH and IgL chains are successfully rearranged, a complete BCR is expressed on the surface of immature B cells, which consists of IgM (with μ IgH and IgL chains) and the signaling components Igα and Igβ. Immature B cells are tested for self-reactivity, and those that bind strongly to self-antigens either undergo receptor editing (to further rearrange the IgL chain) or are eliminated by apoptosis to prevent autoimmune responses, thereby establishing central B cell tolerance [20,30]. Most immature B cells egress the BM and complete their development in SLOs like the spleen. Some authors consider this the transitional phase of immature B cells, while others refer to it as a distinct intermediate stage: the transitional B cells [31]. In this work, immature B cells are defined as BM IgM^{+} IgD^{-} cells, and transitional B cells as IgM^{+} IgD^{low} cells that egress from the BM for further development. Regardless of terminology, maturing B cells begin to express surface IgD in addition to IgM, generated by alternative splicing of RNA transcripts from the IgH locus [32].

In the BM, C-X-C motif chemokine ligand 12 (CXCL12, also known as stromal cell-derived factor 1, SDF-1)-guided chemoattraction is important for maintaining B cell retention in their niches during development

[18,33]. The corresponding receptor, C-X-C motif chemokine receptor 4 (CXCR4) is expressed on all BM B cell subsets during development, starting with HSCs [34]. However, CXCL12-mediated B cell adhesion diminishes during development [35,36] and is eventually overcome by sphingosine-1-phosphate-mediated (S1P-mediated) chemoattraction, driving transitional B cells out of the BM into the bloodstream [37,38]. Only 10 to 20 % of transitional B cells successfully reach the spleen [39]. As they continue to develop, these cells are further categorized into T1 and T2 subpopulations, differing in localization, surface phenotype, and functional characteristics [31,40]. Ultimately, transitional B cells mature into either follicular (FO) or marginal zone (MZ) B cells, both of which are naïve mature B cells expressing surface IgM and IgD within a functional BCR complex [19,41]. FO B cells are primarily located in splenic follicles but also circulate in the periphery, including other SLOs and the BM [1]. In contrast, the smaller population of MZ B cells are confined to the spleen's MZ, where they monitor blood-borne antigens [42]. MZ B cells exhibit more innate-like properties than FO B cells, similar to B-1 cells, including a heightened responsiveness to TLR stimulation and rapid, low-affinity IgM production independent of T cell help [42,43].

B-1 cells: B-1 cells, a small B cell subpopulation, have distinct developmental origins, phenotypes, and functions compared to B-2 cells. They are rare in the BM, spleen, and SLOs, where they comprise only about 0.1 to 2 % of the total pan-CD19$^+$ B cell population. However, they are enriched in the peritoneal and pleural cavities, where they constitute 35 to 70 % of the pan-CD19$^+$ B cells [44]. The B-1 cell pool arises from multiple developmental origins, most of which occur during fetal and neonatal stages, prior to the emergence of conventional B cells (which were thus named B-2 cells) [19,45,46]. Although B-1 cells are derived from HSCs, certain aspects of their development remain unclear, with two models being discussed: the lineage model suggests that B-1 cells arise from distinct progenitors [47-53], whereas the selection model proposes that they develop from a common progenitor with B-2 cells, with differentiation driven by BCR antigen recognition [54-59]. Evidence supporting both models has led to the proposal of a hybrid model that incorporates elements from each [44,60].

B-1 cells are further divided into B-1a (CD5$^+$) and B-1b (CD5$^-$) subpopulations, based on the expression of the CD5 (also known as Ly-1) surface marker (Fig.2) [61]. Although these subpopulations share a similar surface phenotype and the capacity for self-renewal, they perform distinct immune functions. B-1a cells have a more restricted receptor repertoire that is biased toward recognizing bacterial and self-antigens [44,58,60,62]. They are recognized as more innate-like B cells and are key producers of natural polyreactive IgM antibodies [42,44]. However, B-1a cells do not generate immune memory and are prone to autoantibody production, potentially contributing to autoimmune diseases [44,61,63]. In contrast, B-1b cells are primary sources of rapid T cell-independent antibody responses to pathogens, and unlike B-1a cells, they can contribute to long-term protection by establishing immune memory [64-66].

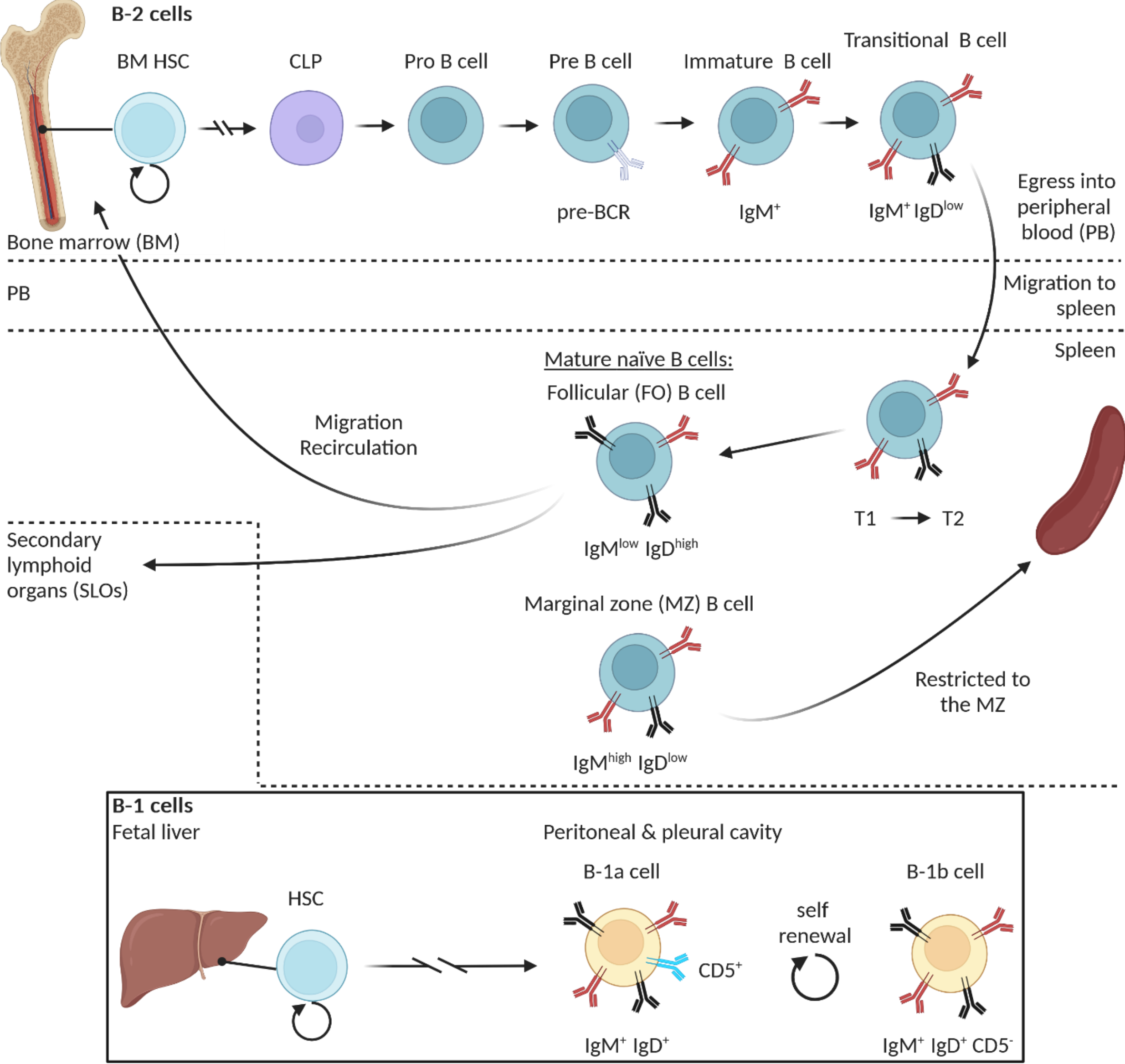


Figure 2: B cell development. B-2 cell development begins in the BM with hematopoietic stem cells (HSCs), which progress through several intermediate stages to differentiate into common lymphoid progenitors (CLPs). CLPs give rise to pro-B cells, where genetic recombination of the V, D and J loci initiates the formation of the immunoglobulin heavy chain (IgH). As pro-B cells transition into pre-B cells, the functionality of the IgH is tested within a pre-B cell receptor (BCR) complex. Successful signaling from the pre-BCR allows survival and progression to the rearrangement of the Ig light chain (IgL). Immature B cells are the first stage to express a complete BCR on their surface, consisting of IgM. In the transitional B cell stage, alternative mRNA splicing enables the expression of low levels of surface IgD in addition to IgM. Transitional B cells egress into the peripheral blood (PB) and migrate to the spleen, where they mature through the T1 and T2 stages into naïve mature B cells. T2 cells differentiate into either follicular (FO) B cells (IgM^{low} IgD^{high}), which perform conventional adaptive immune functions, or marginal zone (MZ) B cells, which exhibit innate-like functions, including rapid and T cell-independent IgM secretion, similar to B-1 cells. FO B cells reside in splenic follicles but also circulate through secondary lymphoid organs (SLOs) and the BM. In contrast, MZ B cells are non-circulating and localized predominantly in the splenic MZ. Most B-1 cells are of fetal origin, where development begins with HSCs in the fetal liver. In adult mice, B-1 cells are rare in the spleen and other SLOs but enriched in the peritoneal and pleural cavities. These cells are further divided into two subpopulations: B-1a ($CD5^+$) and B-1b ($CD5^-$) cells. Unlike B-2 cells, which require continuous replenishment through *de novo* development in the BM, B-1 cells possess self-renewing capacities. The developmental mechanism(s) of B-1a and B-1b cells remain under investigation, with evidence supporting both lineage and selection models. Created with BioRender.com.

1.1.3 Activation of mature B cells and the generation of high-affinity antibodies in germinal centers

The mechanisms of B cell activation vary depending on the B cell subpopulation (FO, MZ, or B-1 cells), the nature of the antigen (T cell-dependent [TD] vs. T cell-independent [TI]), and factors such as receptor signaling pathways and cytokines. MZ and B-1 cells are particularly adept at responding to TI antigens, including bacterial components, due to their elevated TLR expression and BCR repertoire, which is skewed toward recognizing conserved microbial elements. This rapid, $CD4^+$ T helper-independent response primarily results in the secretion of unmutated, low-affinity antibodies [42,43,62,67,68]. In contrast, TD activation of B cells, though slower, produces high-affinity, hypermutated, and class-switched antibodies (Fig. 3). Under steady-state conditions, FO B cells and T cells reside in distinct regions within the spleen and lymph nodes (LNs) known as follicles and T cell zones, respectively [69]. DCs in these areas aid in activating both B and T cells by presenting antigens. BCR recognition of an antigen triggers signaling cascades that lead to antigen internalization, processing, and presentation on MHCII molecules on the B cell surface. This also promotes B cell migration to T cell-rich zones within SLOs, where activated T_{FH} cells interact with B cells *via* MHCII-bound antigens and provide co-stimulation *via* CD40-CD40L interactions and cytokine secretion [70].

Following activation and clonal expansion, some B cells form primary foci at the T/B border and differentiate into short-lived plasmablasts, an intermediate stage toward fully differentiated plasma cells (PCs). These plasmablasts, as part of the extrafollicular antibody response, predominantly secrete low-affinity IgM and typically undergo apoptosis within 3 – 5 days [1,71-73]. Meanwhile, other activated B cells with relatively high-affinity BCRs are selected to enter the GC reaction and migrate back into the follicle. Within the GC, which is divided into a dark zone (DZ) and light zone (LZ), BCR genomic loci undergo somatic hypermutation (SHM), and B cells are subjected to affinity selection and potentially perform class-switch recombination (CSR). The DZ is densely packed with activated B cells, now called centroblasts, which proliferate rapidly and undergo SHM [1,74,75]. During SHM, the enzyme activation-induced cytidine deaminase (AID) introduces mutations in the variable regions of the IgH and IgL loci, potentially altering the antigen affinity of the resulting BCRs [76]. The mutated B cells then migrate to the LZ as centrocytes, where their new BCRs are tested for affinity by interacting with antigens displayed on follicular DCs (FDCs). High-affinity centrocytes receive additional co-stimulatory signals from T_{FH} cells, which evaluate antigen presentation *via* MHCII [75,77,78]. Positively selected centrocytes re-enter the DZ for further rounds of SHM and selection, whereas low-affinity variants do not receive T_{FH}-help and undergo apoptosis. The recurring migration of GC B cells between the DZ and LZ compartment is also known as cyclic re-entry [75,79]. T_{FH}-derived signals can also initiate CSR, allowing the antibody isotype to switch from IgM to IgG, IgA, or IgE [1]. While CSR was traditionally thought to occur primarily in the

GC, recent evidence suggests it is already induced during early T-B cell interactions at the T/B border, before GC entry [80]. Ultimately, after affinity maturation and, in some cases, CSR, GC B cells differentiate into either long-lived PCs or memory B cells. PCs often migrate to the BM for sustained antibody production [73], while memory B cells remain primed to respond rapidly to future infections with the same pathogen, thereby contributing to long-term immunity [1].

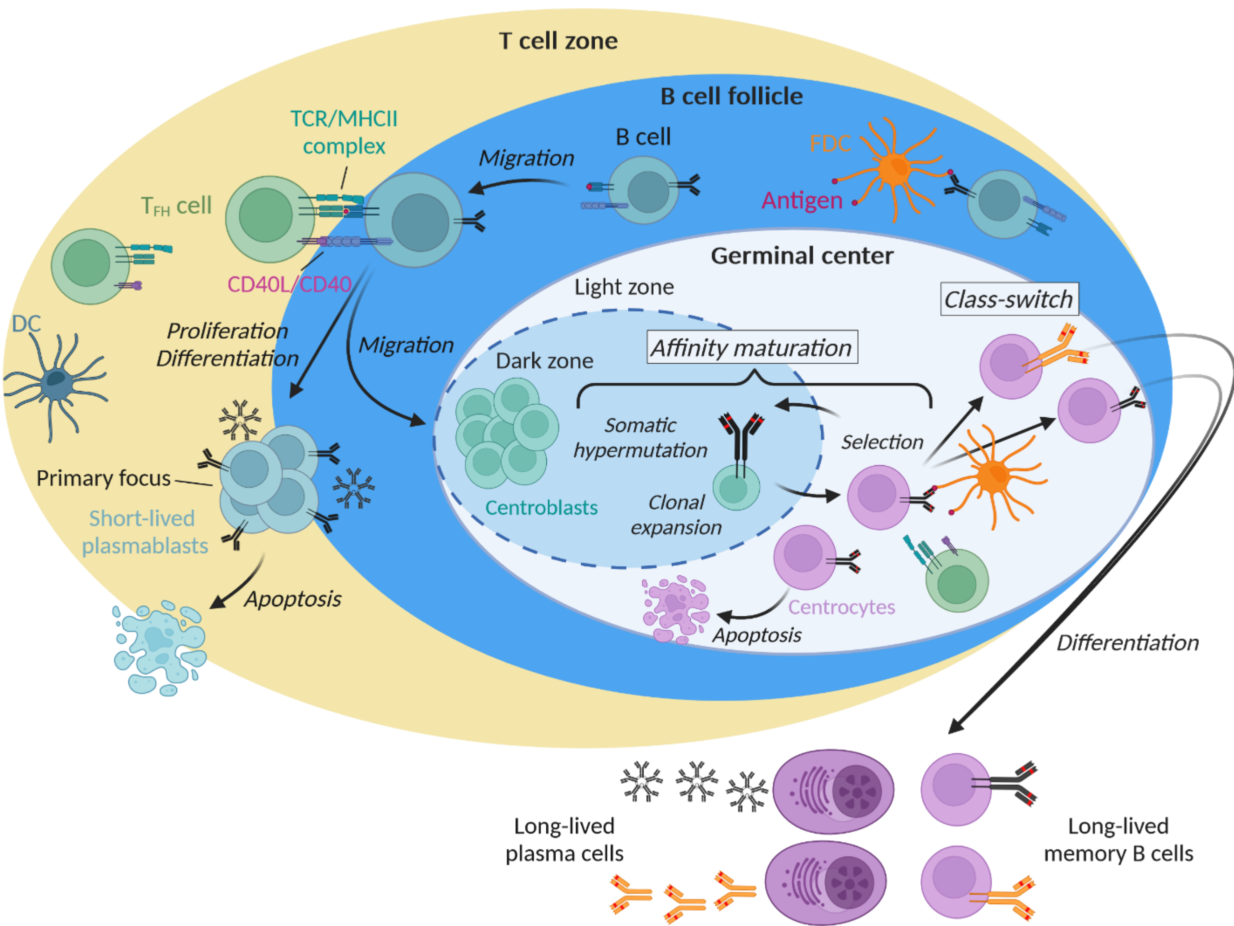


Figure 3: T cell-dependent generation of high-affinity, class-switched antibodies during the GC reaction. The GC reaction begins with the activation of naïve B cells by follicular DCs (FDCs), which present antigens and direct B cell migration to the T/B border. Similarly, naïve $CD4^+$ T cells are activated by DCs presenting antigens *via* MHCII, initiating their differentiation into T follicular helper (T_{FH}) cells and migration to the T/B border. Here, activated B and T_{FH} cells engage in bidirectional signaling: B cells present antigen *via* MHCII, reinforcing T_{FH} commitment, while T_{FH} cells provide co-stimulation through CD40L and cytokine secretion, promoting B cell survival and differentiation. Some activated B cells form a primary focus near the T/B border, where they undergo clonal expansion and differentiate into plasmablasts, leading to an early secretion of predominantly low-affinity IgM. Most of these plasmablasts are short-lived and undergo apoptosis within 3 to 5 days. Meanwhile, other activated B cells migrate back into the follicle, where they form the germinal center (GC), which is divided into a dark zone (DZ) and a light zone (LZ). In the DZ, B cells, now called centroblasts, proliferate rapidly and undergo somatic hypermutation (SHM). This process introduces mutations in the variable regions of the BCR's IgH and IgL, potentially altering antigen affinity. Following SHM, mutated centroblasts migrate to the light zone (LZ) and become centrocytes, and their altered BCRs are tested against antigens presented by FDCs. High-affinity variants receive survival and proliferation signals from T_{FH} cells, which assess B cell antigen presentation *via* MHCII. This affinity-based selection ensures the survival of high-affinity variants, which re-enter the DZ for further rounds of SHM and selection. This iterative cycle drives BCR affinity maturation, a critical step in the generation of high-affinity antibodies. Additionally, some GC B

cells undergo class-switch recombination (CSR), which changes the antibody isotype from IgM to IgG, IgA, or IgE, enhancing functional versatility. Ultimately, GC B cells differentiate into either long-lived plasma cells (PCs), which migrate to the BM to sustain antibody production, or memory B cells, which remain primed for rapid responses to future infections, providing long-term immunity. Created with BioRender.com.

1.2 Toxoplasma gondii

Toxoplasma gondii is an obligate intracellular eucaryotic parasite belonging to the phylum Apicomplexa. It can infect virtually all warm-blooded animals, including humans, and is the causative agent of toxoplasmosis, a disease that can result in severe complications, particularly in immunocompromised individuals or if primary infection occurs during pregnancy [81-83]. *T. gondii* is categorized into three major clonal lineages or strains: type I, type II, and type III [84,85]. Type I strains are more virulent than type II and III strains, causing higher mortality rates in infected mice [86]. Although type II is the most common strain among animals and humans, type I strains are often found in immunocompromised patients [85,87,88]. With no available vaccine for humans [89] and approximately 30% of the global human population infected [90], *T. gondii* poses a serious public health concern.

1.2.1 Life cycle and routes of infection

The life cycle of *T. gondii* (Fig. 4) alternates between sexual and asexual reproduction, depending on the host species [91]. In intermediate hosts, such as all warm-blooded animals, including humans, the parasite undergoes asexual reproduction. Sexual reproduction, however, is restricted to the small intestine of felids (domestic and wild cats), which serve as the definitive hosts for *T. gondii*. This specificity is attributed to the unique metabolic environment in the feline gut. Cats lack the enzyme delta-6-desaturase, which is required to convert linoleic acid into arachidonic acid. Consequently, cats maintain elevated levels of linoleic acid in their intestines, which is essential for triggering the sexual reproduction of *T. gondii* within their gut epithelium [92]. Following sexual reproduction, oocysts are formed and shed in the cat's feces, which become infectious after one to five days of sporulation and can remain viable in the environment for extended periods [93,94].

When sporulated oocysts containing sporozoites or tissue cysts harboring bradyzoites from infected intermediate hosts are ingested by another intermediate host, *T. gondii* initiates asexual reproduction. The parasite differentiates into tachyzoites, its rapidly dividing form. Tachyzoites invade host cells and reproduce within a specialized compartment known as the parasitophorous vacuole (PV), which is a modified host cell membrane aiding in immune evasion and nutrient adsorption [95]. Following several rounds of replication, the host cell ruptures, releasing new tachyzoites that can infect other cells. This cycle of invasion, replication, and cell lysis (Fig. 5) facilitates the dissemination of *T. gondii* throughout the host, establishing acute infection. Eventually, under pressure from the host immune system,

tachyzoites convert into bradyzoites, a slow-replicating stage that becomes encapsuled within tissue cysts, preferentially in the brain, muscle, and eyes of intermediate hosts [91]. During this chronic stage, the infection is kept under control but remains unresolved, posing a persistent risk of reactivation and stage conversion back into tachyzoites [90].

Cats can also become infected by consuming sporulated oocysts or tissue cysts from infected prey, such as rodents or birds. The released sporozoites or bradyzoites then complete the sexual replication cycle within the feline gut, forming male and female gametes, which ultimately produce new oocysts. Alternatively, other intermediate hosts perpetuate the asexual cycle after ingestion of sporulated oocysts or tissue cysts. Humans are commonly infected through consumption of oocyst-contaminated water or vegetables, through exposure to oocyst-contaminated cat litter, or through consumption of tissue cyst-contaminated meat from infected livestock [93,96]. Furthermore, congenital infection can occur *via* transplacental transmission if a primary *T. gondii*-infection is acquired during pregnancy [97], leading to stillbirth/miscarriage or disabilities.

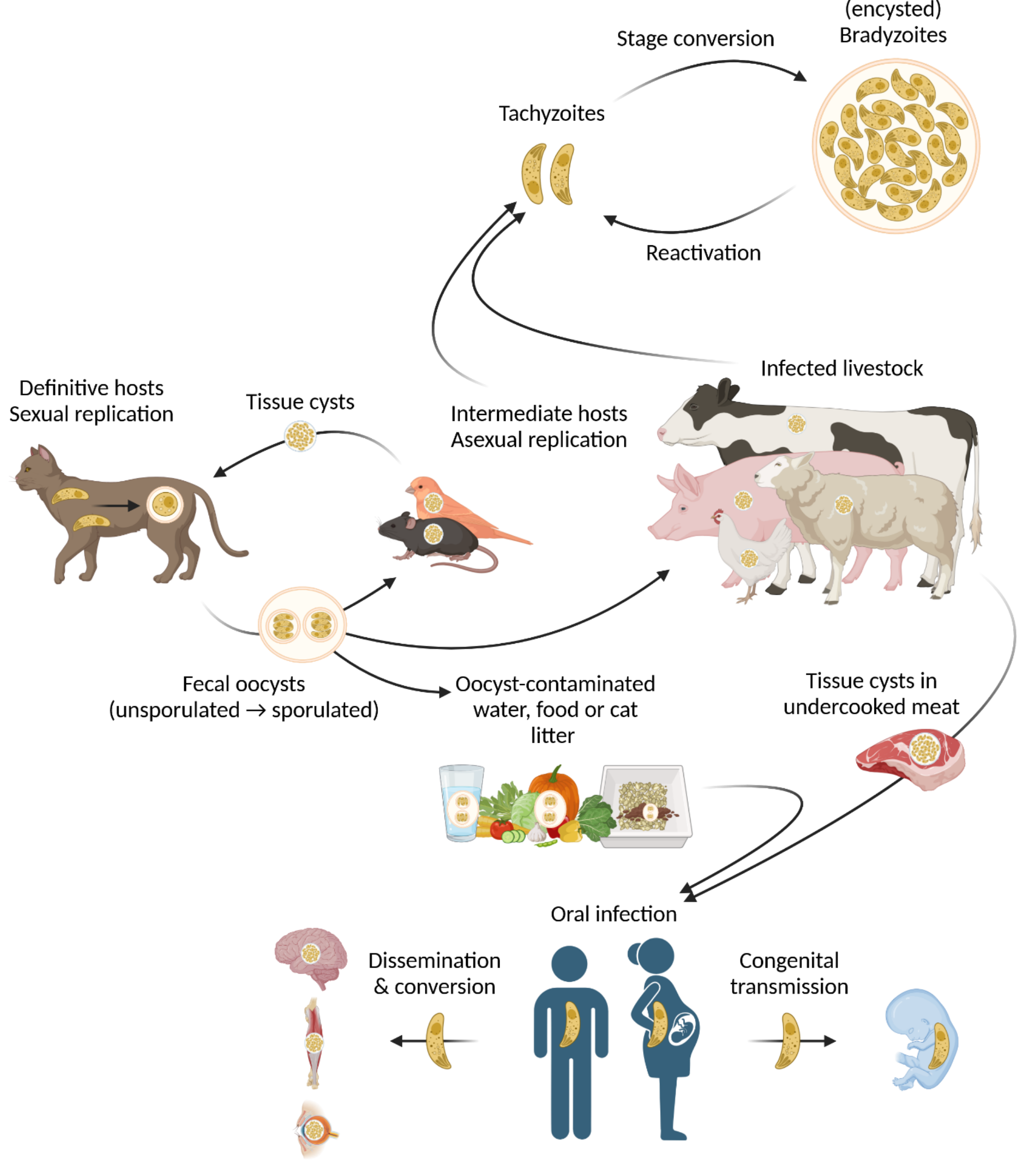


Figure 4: Life cycle and transmission of *T. gondii*. In definitive hosts (e.g. cats), *T. gondii* undergoes sexual replication, resulting in the shedding of fecal oocysts which sporulate and become infectious after one to five days. In intermediate hosts – including rodents and birds, but also livestock and humans – the parasite undergoes asexual replication in the tachyzoite stage. Under immune pressure, the parasite converts into bradyzoites, which persist within tissue cysts and evade the immune system during chronic infection. Reactivation and differentiation back into tachyzoites can occur in individuals with compromised immunity. Definitive hosts are primarily infected by consumption of infected prey, such as rodents and birds. In humans, transmission occurs through the ingestion of oocyst-contaminated food or water, exposure to oocyst-contaminated cat litter, or the consumption of undercooked meat from infected livestock. The parasite spreads inside the host during acute infection and can persist as encysted bradyzoites, preferentially in muscles, the brain or the retina. Congenital transmission can also occur during pregnancy. Created with BioRender.com.

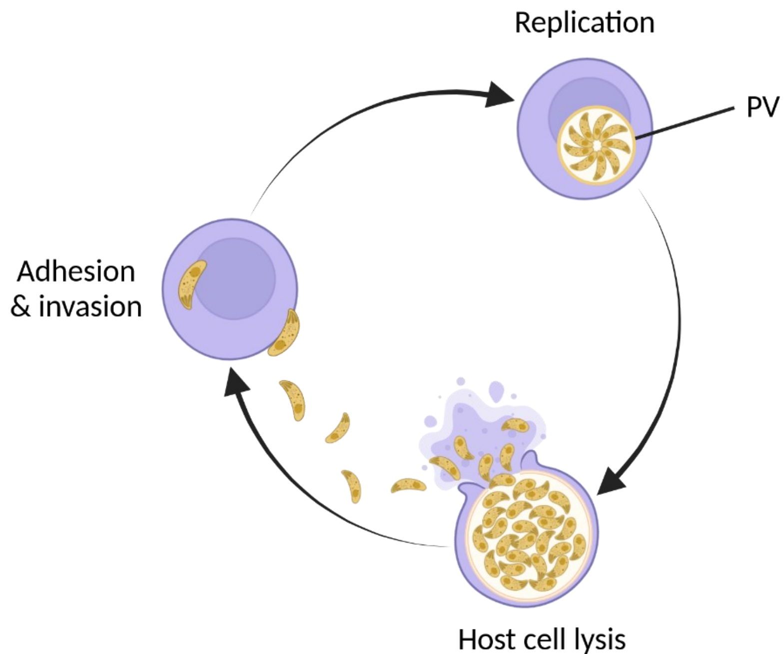


Figure 5: Lytic cycle of *T. gondii*. During asexual replication (acute infection in intermediate hosts), *T. gondii* tachyzoites invade host cells and form a parasitophorous vacuole (PV), which is derived from the host cell membrane and modified to serve as protective niche. Parasitic replication ultimately causes host cell lysis, allowing tachyzoite egress and infection of nearby cells, thereby continuing the lytic cycle. Created with BioRender.com.

1.3 Innate immunity against *T. gondii*

An efficient immune response against *T. gondii* requires coordinated action of innate, adaptive and cell-autonomous immunity, starting with the detection of an ongoing infection. PRRs, such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and Nod-like receptors (NLRs), can recognize parasitic PAMPs (Fig. 6) [98,99]. In mice, *T. gondii* profilin is detected by endosomal TLR11 and TLR12, primarily within $CD8^+$ DCs [100,101]. However, these specific TLRs are absent in humans, raising the question of compensatory molecules or mechanisms involved in *T. gondii* detection [102]. Other TLRs also contribute to pathogen detection; for example, TLR3, TLR7 and TLR9 can sense the presence of parasite-derived nucleic-acids [103], and the C-C-motif chemokine receptor 5 (CCR5) can recognize parasite-derived cyclophilin-18 proteins (TgCyp18) [104-106]. Pathogen recognition by APCs, such as DCs, leads to the

expression of proinflammatory cytokines, especially IL-12, IL-6 and tumor necrosis factor alpha (TNFα), which are crucial for the upregulation of the inflammatory response [99]. IL-12 in particular is essential for the production of interferon-gamma (IFNγ) from NK cells and ILC1s [107-111] and drives the differentiation of naïve $CD4^+$ T cells into T helper type 1 (Th1) cells, another important source of IFNγ during *T. gondii* infection [112-114]. Additionally, $CD8^+$ T cells [115,116], macrophages [117], and neutrophils [118,119] were found to contribute IFNγ in this setting. IFNγ is essential for the induction of cell-autonomous immune responses, which are among the most effective mechanisms for limiting parasite replication and are therefore crucial for the host to gain control over the infection [98,120].

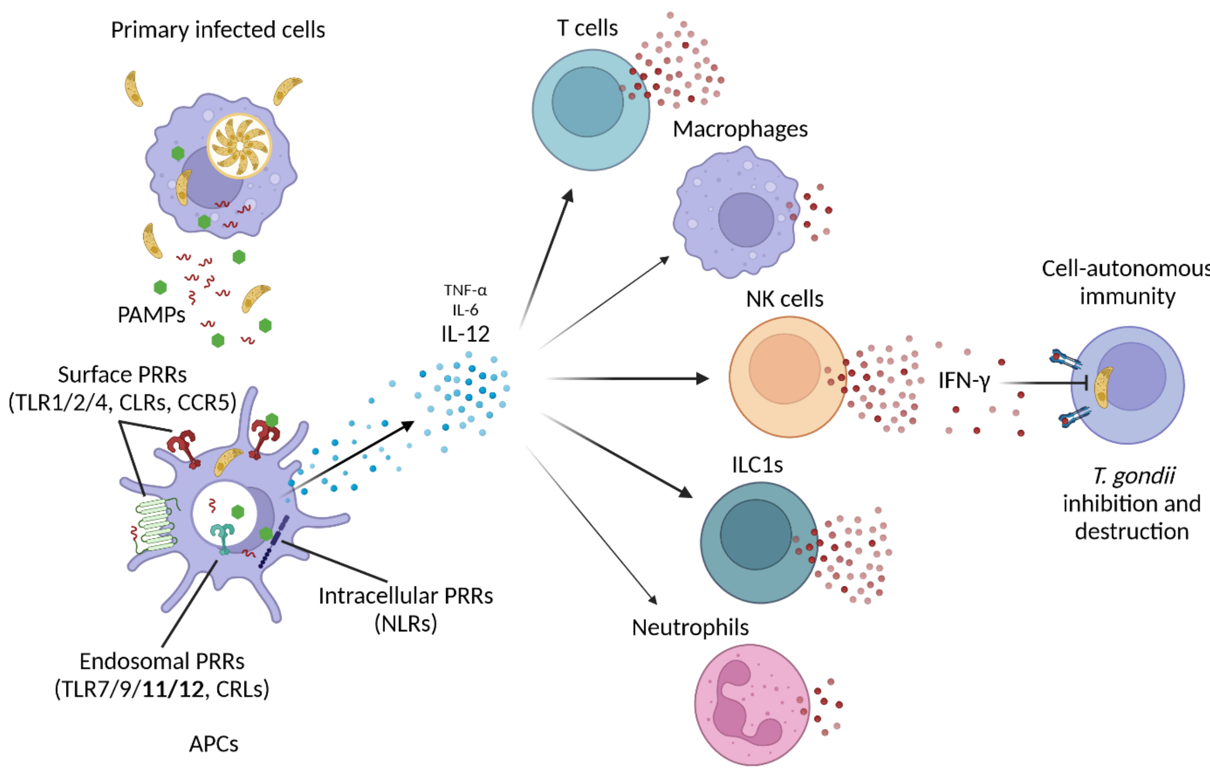


Figure 6: Detection of *T. gondii*-derived molecules and cellular sources of IFNγ. Pathogen-associated molecular patterns (PAMPs) from *T. gondii* are detected by pattern recognition receptors (PRRs) on the surface and within antigen presenting cells (APCs). Relevant PRRs are located in the cell membrane (e.g. Toll-like receptor 1 [TLR1], TLR2, TLR4, C-type lectin receptors [CLRs] and C-C-motif chemokine receptor 5 [CCR5]), in the cytoplasm (e.g. Nod-like receptors [NLRs]) and in the endosomal membrane (e.g. TLR7, TLR9, TLR11, TLR12 and CRLs). Among the most well-known mechanisms is the recognition of *T. gondii* profilin by TLR11 and TLR12. APC activation promotes the expression of proinflammatory cytokines, such as interleukin 12 (IL-12), IL-6 and tumor necrosis factor alpha (TNF-α). IL-12 then drives expression of interferon gamma (IFNγ) from natural killer (NK) cells, innate lymphoid cells type 1 (ILC1s) and $CD4^+$ T cells (after differentiation from naïve into T helper type 1 (Th1) cells). Activated $CD8^+$ T cells, macrophages and neutrophils can also contribute to IFNγ production, albeit to a lesser extent. IFNγ, in turn, induces robust cell-autonomous immunity, which is essential for pathogen inhibition and destruction. Created with BioRender.com.

1.3.1 The Interferon family

The interferon (IFN) family is divided into three classes: type I, type II, and type III, each playing a central role in the immune response against invading pathogens. Among the type I IFNs, IFN-alpha (IFNα) and IFN-beta (IFNβ) are the most well-known and characterized [121]. They signal through a receptor composed of two subunits, IFNAR1 and IFNAR2, activating the Janus activated kinase (JAK)- signal transducer and activator of transcription (STAT) pathway. This cascade results in the formation of phosphorylated STAT-dimers, which form complexes with interferon-regulatory factor 9 (IRF9) and bind to IFN-stimulated response elements (ISREs) in the genome to drive the expression of IFN-stimulated genes (ISGs) [122,123]. IFN-α and IFN-β are produced by a variety of cell types, and while they contribute to innate immune responses against bacterial, parasitic, and fungal infections, they are primarily known for their potent antiviral activity [124].

Type II IFN is represented solely by IFN-gamma (IFNγ), which is structurally distinct from type I and III IFNs [122]. IFNγ signals through a distinct heterodimeric receptor composed of the IFNGR1 and IFNGR2 subunits. Downstream signaling is mediated primarily by phosphorylated STAT1 homodimers that bind to gamma-interferon activation site (GAS) promotor elements, inducing the expression of ISGs [122,125]. During *T. gondii* infection, IFNγ is predominantly produced by NK cells, ILC1 cells, and $CD4^+$ Th1 cells [98,108-111,113,114], while other immune cells can contribute low(er) amounts [115-118]. In addition to its antiviral properties, IFNγ plays a key role in the “priming” of macrophages, promoting their M1-type (proinflammatory) polarization and production of proinflammatory cytokines and reactive oxygen species (ROS) [126,127].

Type III IFNs, also known as lambda-IFNs (IFNλs), are the least-characterized members of the IFN family. While closely related to type I IFNs, they are also structurally related to the IL-10 cytokine family and bind the IL-10R2 subunit, which pairs with the IFNLR1 subunit to form the type III IFN receptor. Although induced through a different receptor, downstream signaling pathways and transcriptional responses of type III IFNs are similar to those of type I IFNs [128]. However, their activity is largely confined to epithelial barrier tissues because the expression of the IFNLR1 receptor subunit is restricted to these cells, resulting in a more localized response compared to the systemic effects of type I IFNs [129].

Notably, while IFNs are primarily associated with proinflammatory roles, they can also exhibit immune-regulatory functions [130].

1.3.2 IFNγ-induced cell-autonomous immunity against *T. gondii*

As mentioned, IFNγ is a critical cytokine in the host defense against intracellular pathogens like *T. gondii*, as it activates hundreds of genes involved in cell-autonomous immunity [99,100], including GTPase family members such as immunity-related GTPases (IRGs, also known as p47 GTPases) and guanylate-binding proteins (GBPs, also known as p65 GTPases). In C57BL/6 mice, 23 IRG genes are located on chromosomes 7, 11 and 18 [131], while 13 murine GBPs (mGBPs) clustered on chromosomes 3 and 5 have been identified [132-134]. Although the roles of individual IRGs and mGBPs vary and certain mechanisms remain unclear, their protective function generally involves the formation of homo- and hetero-oligomers that accumulate at the PV, leading to its disruption and parasite destruction [132,135-138]. The essential role of specific mGBPs has been demonstrated *in vivo*, as mice deficient in multiple or even single mGBPs displayed significantly increased susceptibility to *T. gondii* [137-143]. Additionally, IFNγ-mediated host defense mechanisms aim to “starve” the parasite by depleting essential amino acids like tryptophan and arginine, which are crucial for parasite survival and replication. Tryptophan is depleted by the enzyme indoleamine 2,3-dioxygenase (IDO) [144,145], while arginine is depleted through the inducible nitric oxide synthase (iNOS) [146,147]; furthermore, arginine starvation has been identified as a metabolic trigger for tachyzoite-to-bradyzoite stage conversion [148]. However, the role of IDO during *T. gondii* infection remains somewhat unclear due to conflicting data, which indicate either a significant [149-151] or a negligible [152] contribution to parasite control *in vivo*. In addition to arginine depletion, iNOS generates nitrogen oxide (NO), a toxic molecule with potent antimicrobial properties against a wide range of intracellular pathogens [153-155]. In mice, iNOS plays a rather complex role: it appears negligible during acute *T. gondii* infection but is critical for controlling chronic cerebral toxoplasmosis and preventing toxoplasma encephalitis, especially in C57BL/6 mouse strains [149,156,157]. Similarly, reactive oxygen species (ROS) have been shown to inhibit intracellular parasites both *in vitro* and *in vivo* [158-161], but their role during toxoplasmosis remains ambiguous, as oxidative stress, while effective against the parasite, can also cause severe pathogenesis in the host [162-165]. In summary, IFNγ-mediated effector mechanisms against *T. gondii* include the disruption of the PV and destruction of the parasite by GTPases, the production of toxic molecules such as NO and ROS, and nutrient deprivation through arginine and tryptophan depletion.

1.3.3 *T. gondii* counter measures against innate immune responses

As a result of the ongoing evolutionary arms race between host and pathogen, *T. gondii* and other intracellular parasites have developed strategies to counteract host defense mechanisms, including those driven by IFNγ. These strategies include the release of effector molecules from specialized apical organelles in *T. gondii*, known as rhoptries and dense granules, after host cell invasion [91]. For example,

the secreted dense granule protein Toxoplasma gondii inhibitor of STAT1 transcription (TgIST) disrupts both type I and II IFN-mediated gene expression by binding phosphorylated STAT-dimers in the nucleus. This binding blocks gene transcription by preventing the recruitment of co-transcriptional activators and instead recruiting the suppressive nucleosome remodeling and deacetylase (NuRD) complex [166-170]. As a result, the expression of downstream effector molecules, such as iNOS [171] and IDO [172], is reduced in *T. gondii*-infected cells, which inhibits IFN-mediated immune responses. However, TgIST is just one example. The *T. gondii* genome encodes a variety of rhoptry proteins (ROPs) and dense granule proteins (GRAs), and while several have been well-characterized, the functions of others remain unknown. These parasite-derived effector molecules are key virulence factors and major determinants in the classification of *T. gondii* strains into types I, II and III, significantly impacting their pathogenicity [173,174]. For instance, ROP5, ROP17, ROP18 and GRA7 subvert the recruitment of IRGs and mGBPs to the PV, underscoring the importance of this defense mechanism and the parasites need to interfere with it [173-180]. Additionally, GRA28 promotes the migration of *T. gondii*-infected macrophages by inducing transcriptional remodeling that drives the expression of CCR7, aiding in parasite dissemination within the host [181]. *T. gondii* and other apicomplexan parasites can also evade the complement system, a key prerequisite for establishing infection [182,183]. Furthermore, *T. gondii* can prevent or initiate host cell apoptosis depending on the parasite strain, cell type and infection stage [184,185]. In summary, while the evolutionary arms race between host and parasite has driven the development of a robust innate immune response in hosts, the parasite has countered with sophisticated immune evasion strategies.

1.4 The lymphotoxin β receptor (LTβR) and the lymphotoxin signaling network

The lymphotoxin β receptor (LTβR) is a member of the tumor necrosis factor (TNF) / TNF-receptor superfamily (TNFRSF), which consists of 29 receptors and their 19 cognate ligands [186]. Most TNFRSF members are type I transmembrane glycosylated proteins with extracellular domains containing between one and six cysteine-rich domains (CRD), which are a hallmark of the TNFRSF and confer ligand specificity and affinity [187,188]. On the other hand, the TNF superfamily ligands are, in general, type II transmembrane proteins with intracellular N-termini and extracellular C-termini, which self-assemble into (mostly) homotrimers [187]. This receptor-ligand superfamily forms a complex communication network, as many receptors can bind multiple ligands, and *vice versa*. It regulates various biological processes such as organogenesis, cell death, host defense, inflammation, and autoimmunity [187-189].

The LTβR is present on most cell types, including stromal fibroblasts, endothelial-, epithelial-, myeloid- and dendritic cells, but it is notably absent from lymphoid cells [187,190-197]. In contrast, the two known ligands of the LTβR are expressed primarily on lymphoid cells. These ligands are (1) the membrane-bound lymphotoxin (LT) heterotrimer named LT$\alpha_1\beta_2$ and (2) LIGHT (**l**ymphotoxin-like, exhibits **i**nducible

expression, and competes with HSV **g**lycoprotein D for **h**erpesvirus entry mediator, a receptor expressed by **T** lymphocytes), which exists in both membrane-bound and shed forms [198,199]. LIGHT is also expressed by inflammatory effector cells such as macrophages, neutrophils, and DCs, but not by naïve and regulatory T cells (LIGHT expression is induced in activated effector and memory $CD4^+$ and $CD8^+$ T cells) as well as B cells. This expression pattern suggests that LIGHT plays a key role in acute inflammatory and adaptive immune responses [200].

While TNFRSF-mediated signaling typically requires ligand-induced receptor trimerization, sufficient LTβR activation by $LT\alpha_1\beta_2$ is already achieved through receptor dimerization [188,201]. Downstream signaling is mediated by the recruitment of TNFR-associated factors (TRAFs) to TRAF-interacting motifs (TIMs) within the LTβR intracellular domain. Of the six typical TRAF (TRAF1-6) family members, TRAF2, TRAF3, and TRAF5 are the primary adaptor proteins recruited upon LTβR activation. TRAF7 is considered atypical due a non-canonical TRAF domain [202,203]. The nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) pathway, activated through TRAF2 and TRAF5 [204-206], and the c-Jun N-terminal kinase (JNK) pathway, activated through TRAF3 [207-211], are two major intracellular signaling pathways downstream of the LTβR (Fig. 7) [187,188,212]. The biological outcome of LTβR signaling depends on the ligands, adaptor proteins, and the cell's biological state. NF-κB activation promotes organogenesis, immune homeostasis, and inflammation, whereas JNK activation induces apoptosis and cell death; crosstalk between these pathways fine-tunes cellular responses. For instance, NF-κB activation, particularly *via* the canonical pathway, antagonizes JNK-induced apoptosis by promoting pro-survival signals [213], whereas TRAF3 and JNK activation inhibit NF-κB signaling and outcomes [214-217].

The LTβR and its ligands are components of a lymphotoxin signaling network with shared ligands and intersecting downstream signaling pathways (Fig. 7). This network includes other TNFR family members, such as TNFR1 (p55), TNFR2 (p75), herpes virus entry mediator (HVEM), and soluble decoy receptor 3 (DcR3). TNFR1, which contains a death domain (DD) instead of a TRAF binding site, belongs to the death receptor subfamily of TNFRs. Upon binding either soluble (sTNFα) or membrane anchored TNFα (mTNFα), TNFR1 recruits the adaptor protein TNFR type 1-associated death domain (TRADD). TRADD can then activate either the canonical NF-κB pathway or pro-apoptotic pathways, depending on the additional cofactors recruited [187,212,218]. In contrast, TNFR2 and HVEM contain TRAF binding sites (two and one, respectively) and directly interact with TRAF family members to activate JNK and NF-κB [212,219]. TNFR2 preferentially binds mTNFα and, unlike HVEM, can also activate the alternative NF-κB pathway [220,221].

While $LT\alpha_1\beta_2$ is specific for LTβR, LIGHT additionally binds HVEM and DcR3; the latter lacks any direct signaling capability and regulates LIGHT-dependent pathways by limiting LIGHT's bioavailability [200]. LTα, on the other hand, can assemble into either soluble homotrimers (LTα3) or an alternative membrane-anchored heterotrimeric complex ($LT\alpha_2\beta_1$). However, $LT\alpha_3$ does not bind LTβR, and $LT\alpha_2\beta_1$,

which constitutes only a minor fraction of total LTβ complexes, binds LTβR with low affinity [194,222]. Instead, $LT\alpha_3$ and $LT\alpha_2\beta_1$ preferentially bind TNFR1 and TNFR2 [188,212]. In summary, LTβR engagement by its ligands, $LT\alpha_1\beta_2$ and LIGHT, recruits specific TRAF adaptor proteins to activate canonical and alternative NF-κB or JNK signaling pathways. LIGHT also binds HVEM and DcR3, while $LT\alpha_3$ exclusively and $LT\alpha_2\beta_1$ predominantly interacts with TNFR1 and TNFR2 rather than LTβR, forming an interconnected lymphotoxin signaling network (Fig. 7).

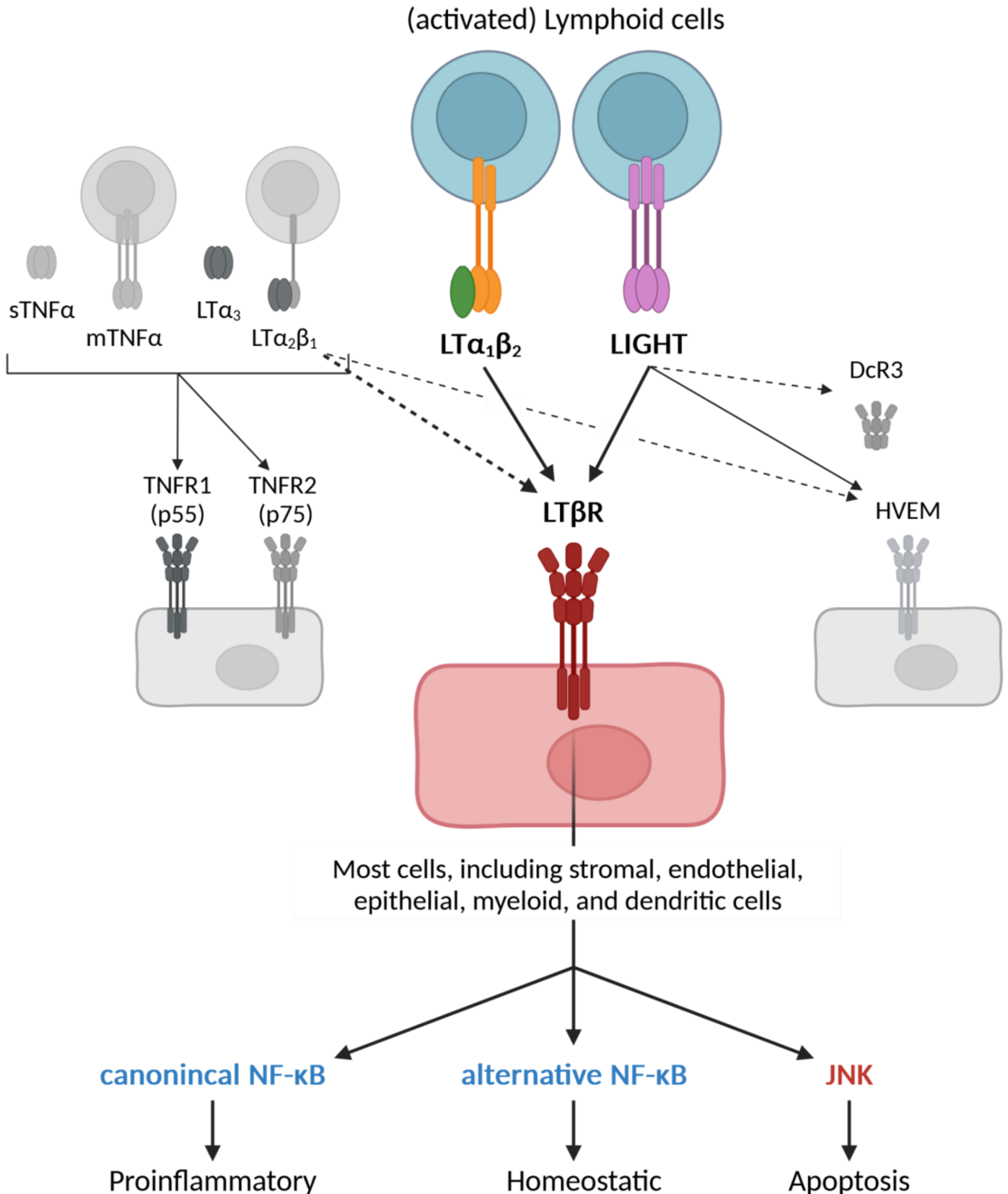


Figure 7: The LTβR, its ligands, and the lymphotoxin signaling network. The LTβR is present on most cell types, including stromal fibroblasts, endothelial-, epithelial-, myeloid- and dendritic cells, but it is absent from lymphoid cells. In contrast, its two primary ligands – membrane-bound $LT\alpha_1\beta_2$ (a lymphotoxin (LT) heterotrimer) and LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for herpesvirus entry mediator, a receptor expressed by T lymphocytes), which exists both as membrane-bound and soluble form – are predominantly found on (activated) lymphoid cells. Signal transduction *via* LTβR engages the canonical nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) pathway, which is mainly associated with

proinflammatory responses, the alternative (non-canonical) NF-κB pathway, which is linked to organogenesis and immune homeostasis, and the c-Jun N-terminal kinase (JNK) pathway, which is associated with apoptosis. The LTβR and its ligands (highlighted in colour) form the core of a broader lymphotoxin signaling network involving additional TNFR receptors and ligands (depicted in gray). While $LT\alpha_1\beta_2$ is LTβR-specific, LIGHT also engages herpesvirus entry mediator (HVEM) and the soluble decoy receptor 3 (DcR3). In contrast, the alternative $LT\alpha_2\beta_1$ binds the LTβR with low-affinity and plays a minor role in LTβR signaling. Instead, $LT\alpha_2\beta_1$ preferentially binds TNFR1 (p55) and TNFR2 (p75), as do the soluble LT homotrimer $LT\alpha_3$, soluble TNFα (sTNFα), and membrane-bound TNFα (mTNFα). Meanwhile, DcR3 functions solely by binding to and neutralizing LIGHT, thereby indirectly modulating LIGHT-dependent pathways. Created with BioRender.com.

1.4.1 The canonical and alternative NF-κB signaling pathways

LTβR signaling can activate both canonical and alternative NF-κB signaling pathways. The mammalian NF-κB family – NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and c-Rel – form structurally related homo- and heterodimeric transcription factors that induce expression of various target genes. Their activity is controlled by inhibitory proteins of the IκB family, which possess characteristic ankyrin repeats that sequester NF-κB dimers in the cytoplasm. The p50 and p52 NF-κB family members are generated from precursor proteins (p105 and p100, respectively), which also function as IκB-like inhibitors due to their ankyrin repeat domains, until they are processed into their active forms [223-225]. The NF-κB pathway has two branches: the canonical pathway, which is activated rapidly but transiently and primarily mediates proinflammatory responses, and the alternative (or non-canonical) pathway, which is slower, more persistent, and mainly involved in organogenesis and immune homeostasis (Fig. 8) [217,223]. The LTβR and TNFR2 are among the few TNFR superfamily members capable of activating both canonical and alternative NF-κB pathways [186,226,227]. Canonical NF-κB signaling is triggered by various stimuli, including PRRs and TNFRs, and requires the proteasomal degradation of IκBα, which normally inhibits the nuclear translocation of canonical NF-κB members in the cytosol, particularly the p50/RelA and p50/c-Rel dimers [225]. This degradation is mediated by the inhibitor of nuclear factor kappa-B kinase (IKK) complex, composed of the two IκB kinases IKKα and IKKβ and the regulatory subunit NF-kappa-B essential modulator (NEMO/IKKγ), leading to the degradation of IκBα and subsequent nuclear translocation of NF-κB dimers to activate canonical pathway target gene expression [228,229].

In contrast, alternative NF-κB activation occurs downstream of specific TNFR superfamily members. Under steady-state conditions, NF-κB-inducing kinase (NIK) is constitutively degraded through the coordinated action of TRAF2 and TRAF3 [230]. Non-canonical NF-κB stimuli lead to TRAF3 degradation, resulting in NIK stabilization and accumulation. NIK subsequently activates IKKα, which induces the processing of p100 by degradation of its IκB-like C-terminal structure, generating active p52/RelB dimers [231,232]. Unlike the constitutive processing of p105 into p50, p100 processing is tightly regulated. The resulting p52/RelB dimers then translocate into the nucleus, where they drive the expression of alternative NF-κB target genes [217,223].

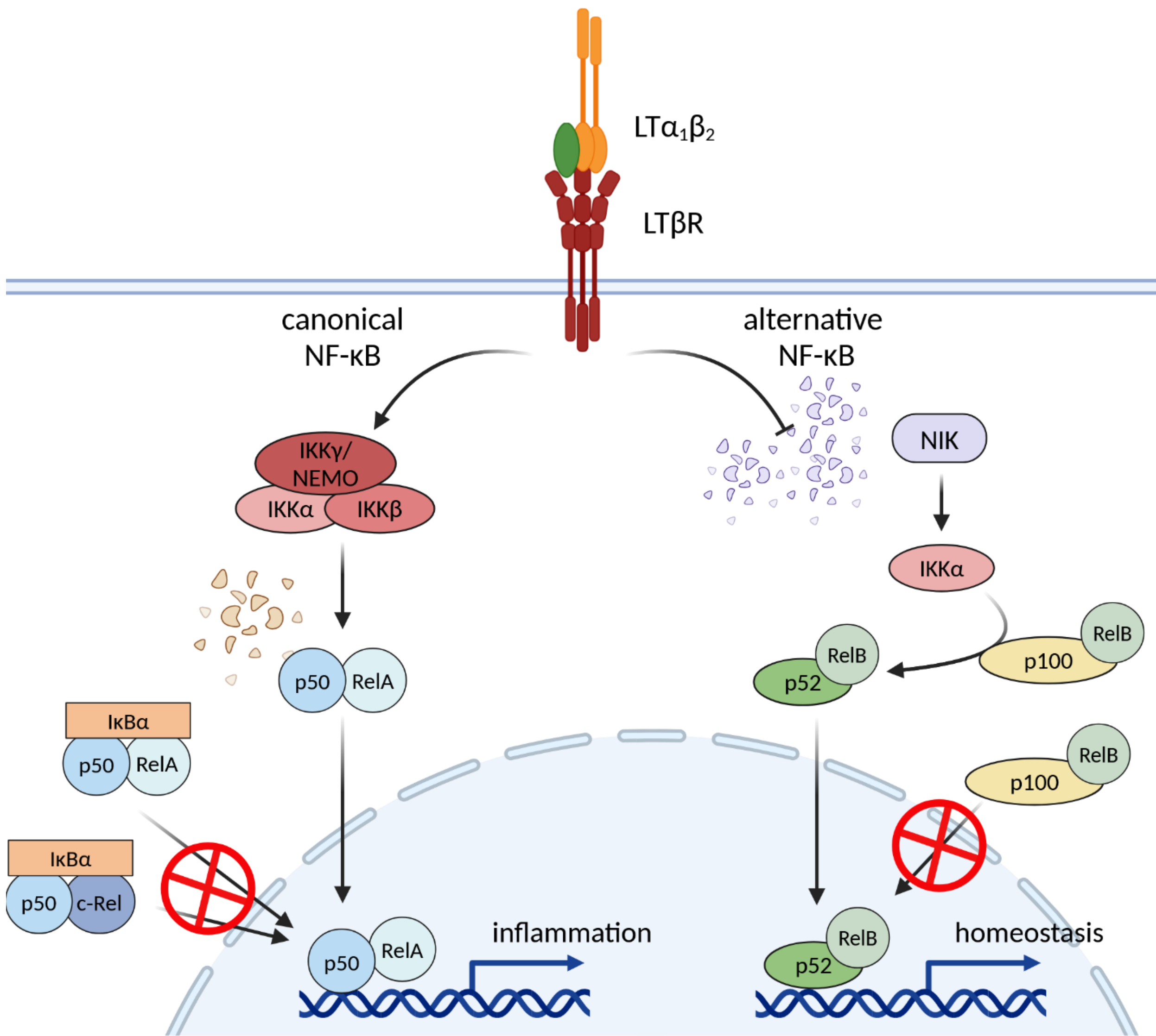


Figure 8: Canonical and alternative NF-κB signaling pathways. Canonical and alternative NF-κB signaling are two distinct pathways downstream of LTβR activation. Canonical NF-κB signaling relies on the formation of the inhibitor of nuclear factor kappa-B kinase (IKK) complex, composed of IKKα, IKKβ, and NF-kappa-B essential modulator (NEMO/IKKγ). This complex promotes the proteasomal degradation of IκBα, which otherwise inhibits the nuclear translocation of NF-κB dimers, such as p50/RelA and p50/c-Rel. Upon IκBα degradation, the freed dimers translocate into the nucleus to induce canonical target gene expression, primarily driving pro-inflammatory responses. In contrast, alternative NF-κB signaling is normally suppressed by the constant degradation of NF-κB-inducing kinase (NIK). LTβR signaling stabilizes NIK, allowing its accumulation and subsequent activation of IKKα. In this pathway, the nuclear translocation of p100/RelB dimers is blocked by the IκB-like C-terminal structure of p100. Activated IKKα processes p100 into p52, generating the active p52/RelB dimer, which translocates into the nucleus to regulate alternative NF-κB target gene expression. Unlike the rapid activation of the canonical pathway, alternative NF-κB signaling is slower and is primarily associated with organogenesis and tissue homeostasis. Created with BioRender.com.

1.4.2 LTβR-deficient (LTβR$^{-/-}$) mice

LTβR signaling is essential for the development and maintenance of SLOs and various immune cell populations. Accordingly, LTβR-deficient (LTβR$^{-/-}$) mice exhibit various immunological deficiencies,

including the complete absence of lymph nodes (LNs) and gut-associated lymphatic tissue (GALT), such as Peyer's Patches (PPs) and mesenteric lymph nodes (MLNs) [233,234]. The spleen of LTβR$^{-/-}$ mice is enlarged and displays a disrupted microarchitecture, with absent follicular dendritic cell (FDCs) networks, marginal zone (MZ) structures, and proper B and T cell compartmentalization [196,233,235-243]. LTβR signaling is also essential for the development and homeostasis of DCs [196,244], NK cells and NKT cells [245-248]. In the thymus, LTβR signaling regulates T cell development by guiding thymocyte trafficking, likely *via* expression of adhesion molecules and chemokines; it also plays a role in central tolerance induction and the regeneration of thymic damage [249]. Furthermore, LTβR$^{-/-}$ mice show signs of autoimmunity, characterized by splenomegaly, structural thymic alterations, autoantibody production, and inflammatory infiltration of peripheral organs such as the lung and liver, where infiltrates predominantly consist of CD4^{+} T cells and B cells [233,234,242,249-253].

While many of the defects observed in LTβR$^{-/-}$ mice arise during embryonal and neonatal development, LTβR signaling remains crucial for maintaining immune homeostasis in adulthood [234]. Conditional deletion of LTβR in adult mice (LTβR$^{\Delta/\Delta}$, as described in [234]) results in reduced size and disorganization of LNs and PPs, with fewer FDCs and poorly defined B and T cell areas compared to wild-type (WT) mice. The spleen of LTβR$^{\Delta/\Delta}$ mice is similarly disrupted, although not as severe as in LTβR$^{-/-}$ mice. Moreover, LTβR$^{\Delta/\Delta}$ mice display reduced numbers of neutrophils, NK cells and NKT cells. However, LTβR$^{\Delta/\Delta}$ mice do not exhibit the inflammatory infiltrations of non-lymphoid organs and the impaired thymus structure observed in LTβR$^{-/-}$ mice, which suggests that these abnormalities stem from the absence of LTβR signaling during early development rather than adulthood [234]. Consequently, due to its essential and multifaceted roles, LTβR signaling continues to garner increasing attention in the study of autoimmune diseases [193,254-256] and cancer [257,258].

1.4.3 LTβR$^{-/-}$ mice during infection

LTβR signaling is also involved in host resistance against various pathogens, as demonstrated for bacteria such as *Listeria monocytogenes* [259-261], *Mycobacterium tuberculosis [259]*, and *Citrobacter rodentium* [234,262,263]; viruses such as *Cytomegalovirus* [264] and Zika virus [265]; and intracellular parasites such as *Leishmania major* [266], *L. donovani* [267], *Plasmodium chabaudi* [268], *P. berghei* [269], and *T. gondii* [242,248], with the latter 3 belonging to the phylum Apicomplexa.

LTβR$^{-/-}$ mice exhibit severely impaired innate and adaptive immune responses. Innate immunity defects include reduced or delayed expression of critical cytokines and effector molecules, such as type I IFNs during murine *Cytomegalovirus* (MCMV) infection [270] and IFNγ and mGBPs during *T. gondii* infection [242,248]. Adaptive immunity is also compromised, as evidenced by reduced secretion of IFNγ from CD4^{+}

T cells and diminished release of cytotoxic granules from $CD8^+$ T cells during *T. gondii* infection [248]. Furthermore, humoral immunity is notably affected, as $LT\beta R^{-/-}$ mice fail to form GC reactions in the spleen [233,271,272]. During *T. gondii* infection, these mice exhibit reduced amounts of parasite-specific IgM and absent parasite-specific IgG in their serum, indicating severe defects in the generation of high-affinity, class-switched antibodies [248]. On the other hand, the loss of LTβR signaling is predicted to increase the expression of genes related to BCR signaling, Ig production, and humoral immunity mediated by circulating Ig, as suggested by a host-pathogen network prediction model based on mRNA sequencing data from lung tissue of WT and $LT\beta R^{-/-}$ mice [248]. These combined deficiencies result in significantly increased mortality in $LT\beta R^{-/-}$ mice, which display increased pathology and parasite burden across multiple organs, including the lung, liver and spleen [242,248]. However, the B cell compartments outside the spleen and LNs of $LT\beta R^{-/-}$ mice, particularly in the BM and peritoneal cavity (PerC), have notably remained poorly characterized until this study.

2. Aim of the thesis

LTβR$^{-/-}$ mice exhibit diverse immunological defects, ranging from impaired development of SLOs to disruptions in immune cell homeostasis and innate and adaptive responses to pathogen infections [233,234,242,248]. Consequently, these mice also show increased susceptibility to *Toxoplasma gondii* infection, marked by significantly increased mortality. A particularly intriguing finding was the reduction of *T. gondii*-specific IgM and the complete absence of *T. gondii*-specific IgG in the serum of infected LTβR$^{-/-}$ mice, which indicate defects in affinity maturation and immunoglobulin class switching [248]. Furthermore, WT mice exhibit a severe reduction in splenic B cell numbers on day 10 p.i., a phenotype less pronounced in LTβR$^{-/-}$ animals [248]. However, prior to this work, no detailed analysis of B cell subpopulations outside the spleen of LTβR$^{-/-}$ mice had been performed.

The aim of this thesis was to further characterize the role of the LTβR in the immune response, specifically the humoral immune response, against intracellular parasites using a murine *T. gondii* infection model. Thus, immune cell populations, with a focus on B cells, were analyzed *via* flow cytometry in the bone marrow, peritoneal cavity, and blood of *T. gondii*-infected LTβR$^{-/-}$ mice. Furthermore, the LTβR$^{-/-}$ bone marrow transcriptome was sequenced and examined, and a histological study of the murine lung, liver, brain, kidney, spleen and bone marrow during *T. gondii* was conducted. These data were further complemented by evaluations of parasite burden and cytokine concentrations. As a result, novel and distinct alterations in various immune cell (sub)populations across multiple compartments of LTβR$^{-/-}$ mice were identified, with findings from the transcriptome and histological analysis aligning closely with flow cytometry results (this study) and data from our previous studies [242,248].

To date, it has not been possible to develop a human-approved vaccine against *T. gondii*. The prevalence of *T. gondii* in the human population and its impact on global health underscore the importance of achieving effective antibody-mediated protection against this parasite. Therefore, a passive immunization approach was employed to assess the extent of protection provided by transferring immune serum containing antibodies against *T. gondii* into LTβR$^{-/-}$ mice. Mouse survival, parasite burden, splenic immune cell populations, cytokine concentrations, and *T. gondii*-specific antibody concentrations in the serum before and after transfer were evaluated.

Finally, the use of mouse strains with targeted LTβR inactivation in DCs and myeloid cells enables the exploration of potential cell type-specific roles of the LTβR, an investigation that was initiated in this study and should be continued in future studies. Overall, this work was undertaken to broaden our understanding of both LTβR- and *T. gondii*-related immunity and explore new directions for future research.

3. Publications

3.1 Lymphotoxin Beta Receptor$^{-/-}$ Mice Display Altered B and T Cell Subpopulations in the Bone Marrow and Peritoneal Cavity after *Toxoplasma gondii* Infection

(manuscript in revision)

3.2 Antibody-mediated Protection in Lymphotoxin β Receptor-deficient Mice Against *Toxoplasma gondii*

(manuscript in preparation)

3.3 Lymphotoxin β Receptor: a Crucial Role in Innate and Adaptive Immune Responses against *Toxoplasma gondii*

(Infection and Immunity, 2021, vol. 89)

3.1 Lymphotoxin Beta Receptor$^{-/-}$ Mice Display Altered B and T Cell Subpopulations in the Bone Marrow and Peritoneal Cavity after *Toxoplasma gondii* Infection

Authors

Marcel Helle, Ursula R. Sorg, Johannes Ptok, Rachel E. Thomas, Katharina Pracht, Patrick Petzsch, Alain de Bruin, Hans-Martin Jäck, Karl Köhrer, Daniel Degrandi, Klaus Pfeffer

Published in

Manuscript in revision

Impact factor

-

Own contributions to this work

Overall: 70%

Conducted all of the following experiments:

T. gondii infection experiments and sample preparation, flow cytometry, cytokine quantification, qRT-PCRs (parasite load)

Other major contributions:

Design of experiments, manuscript preparation, data analysis

Lymphotoxin Beta Receptor$^{-/-}$ Mice Display Altered B and T Cell Subpopulations in the Bone Marrow and Peritoneal Cavity after *Toxoplasma gondii* Infection

Marcel Helle[1], Ursula R. Sorg[1], Johannes Ptok[2], Rachel E. Thomas[3], Katharina Pracht[4], Patrick Petzsch[5], Alain de Bruin[3], Hans-Martin Jäck[4], Karl Köhrer[5], Daniel Degrandi[1], Klaus Pfeffer[1*]

[1]Institute of Medical Microbiology and Hospital Hygiene, Heinrich Heine University, Düsseldorf, Germany

[2]Institute of Virology, Heinrich Heine University, Düsseldorf, Germany

[3]Department Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

[4]Division of Molecular Immunology, Internal Medicine III, University Hospital Erlangen, Nikolaus-Fiebiger Center, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany

[5]Genomics and Transcriptomics Laboratory, Biological and Medical Research Centre (BMFZ), Heinrich Heine University, Düsseldorf, Germany

*: To whom correspondence should be addressed

Prof. Dr. Klaus Pfeffer

Institute of Medical Microbiology and Hospital Hygiene

Heinrich Heine University Düsseldorf

Universitätsstraße 1

40225 Düsseldorf, Germany

Phone: +49 (0) 211 81-12459

Fax: +49 (0) 211 81-15906

Email: klaus.pfeffer@hhu.de

Keywords: lymphotoxin beta receptor, *Toxoplasma gondii*, immunity, adaptive immune response, B cells, T cells, infection, mouse

Abstract

LTβR-deficient (LTβR$^{-/-}$) mice exhibit severe defects in innate and adaptive immunity against various pathogens, including *Toxoplasma gondii*. Intriguingly, LTβR$^{-/-}$ mice show deficiencies in anti-*Toxoplasma* immunoglobulin (Ig) production and Ig class switching. In this study, we investigated bone marrow (BM) and peritoneal cavity (PerC) immune cell populations of LTβR$^{-/-}$ mice during *T. gondii* infection. Strikingly, LTβR$^{-/-}$ BM exhibited elevated frequencies of mature B cells post-infection. Early B cell differentiation stages were severely reduced in both genotypes due to inflammation-induced CXCL12 downregulation, whereas LTβR$^{-/-}$ mature B cells resisted this reduction, suggesting an altered microenvironment in absence of LTβR signaling. LTβR$^{-/-}$ BM plasma cells (PCs) were mainly surface IgM^{+}, while IgA^{+} PCs were virtually absent; IgM^{-} IgA^{-} (IgG) PC frequencies were comparable to the WT. In the PerC of LTβR$^{-/-}$ mice, B-2 and B-1b cell frequencies were increased, whereas B-1a frequencies remained comparable to WT counterparts, resulting in a distorted B-1a to B-1b cell ratio in LTβR$^{-/-}$ mice. T cell subpopulations were also altered in LTβR$^{-/-}$ BM, with decreased double-negative (CD4^{-}/CD8^{-}) and increased CD4^{+} and CD8^{+} T cell frequencies. Transcriptomic analysis of the BM revealed diminished interferon responses but an upregulated TNFα-NF-κB signaling signature, potentially compensating for the absence of LTβR signaling. In summary, this study identified novel and distinct alterations in B and T cell differentiation, kinetics and activation phenotypes in LTβR-deficient mice, suggesting new potential pathways for future research on LTβR signaling in immune cell homeostasis, migration, and pathogen elimination.

Introduction

The lymphotoxin beta receptor (LTβR) is a member of the tumor necrosis factor receptor superfamily (TNFRSF) and is expressed by a wide variety of cell types including epithelial-, endothelial-, stromal- and myeloid cells (e.g. monocytes, macrophages [1,2], neutrophils [1,3], dendritic cells (DCs) [4-6], and mast cells), but is absent from lymphoid cells [7,8]. Conversely, the two known LTβR ligands $LT\alpha_1\beta_2$ and LIGHT (**l**ymphotoxin-like, exhibits **i**nducible expression, and competes with HSV **g**lycoprotein D for **h**erpesvirus entry mediator, a receptor expressed by **T** lymphocytes) are expressed on lymphoid cells, suggesting that LTβR signaling mainly occurs in a paracrine or juxtacrine manner [7,8]. While the lymphotoxin heterotrimer $LT\alpha_1\beta_2$ is LTβR-specific, the second ligand LIGHT can also bind two other TNFSRF members: herpes virus entry mediator (HVEM) and soluble decoy receptor 3 (DcR3) [7-9]. LTβR-induced downstream signaling occurs primarily *via* the canonical and the alternative nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) pathways [10].

The LTβR is essential for the development of lymphoid organs during embryogenesis, and for structural maintenance of lymphoid organs and immune cell homeostasis in adulthood [11,12]. LTβR-deficient mice (LTβR$^{-/-}$) lack lymph nodes (LN) and Peyer's Patches (PPs) [11,12], exhibit reduced numbers of DCs [5,12,13], and have a marked reduction of natural killer (NK) and NKT cells [12,14-17]. Other affected organs include the thymus, where LTβR signaling is involved in thymocyte migration and selection [18], and the spleen, where LTβR deficiency disrupts B and T cell compartmentalization, germinal center (GC) formation, and the homeostasis of follicular DCs (FDCs) and macrophage subpopulations [11,12,19-24]. The well documented inability of LTβR$^{-/-}$ mice to mount adequate immune responses against various pathogens underscores its critical role for immunity [12,17,25-30]. Additionally, LTβR$^{-/-}$ mice exhibit signs of autoimmunity, including splenomegaly, increased perivascular lymphocytic infiltrations of non-lymphoid organs, autoantibody production, and increased baseline immune activation [11,12,17,26,31,32]. Consequently, there has been growing interest in exploring the role of LTβR signaling in infection, autoimmune diseases, and cancer [33-36].

Toxoplasma gondii is an obligate intracellular protozoan parasite that belongs to the phylum Apicomplexa. *T. gondii* can infect virtually all warm-blooded vertebrates, including humans. This successful parasite has infected 30 - 50% of the world's human population [37] and can persist lifelong in the host [38]. While *T. gondii* infection usually causes mild, flu-like symptoms in immunocompetent hosts, an infection of immunocompromised individuals or congenitally infected unborn children can lead to diverse and severe health issues, including encephalitis, myocarditis, pneumonia, and abortion [39]. No vaccine is currently available against *T. gondii* [40].

The host immune response against *T. gondii* requires a coordinated interplay of innate, adaptive, and cell autonomous immunity [41-44]. *T. gondii* infection stimulates a proinflammatory cascade resulting in massive interferon-gamma (IFNγ) production by NK cells [45,46], CD4^{+} and CD8^{+} T cells [45,47-49], and innate lymphoid cells (ILC1s) [50]. An early and potent production of IFNγ is essential for effective parasite control [51,52]. IFNγ (further) activates immune cells such as monocytes, macrophages, DCs, NK- and T cells, and it induces expression of hundreds of genes involved in cell-autonomous immunity to inhibit pathogens [44]. This includes the direct targeting and destruction of *T. gondii* and its surrounding parasitophorous vacuole (PV) by immunity-related GTPases (IRGs) [53-57] and guanylate-binding proteins (GBPs) [58-62]. However, while a strong T-helper 1 (Th1) response driven by IL-12 and IFNγ is necessary for effective parasite restriction, it can also trigger severe immunopathology in the host [63-68].

In addition to classic CD4^{+} and CD8^{+} T cell populations, double-negative (DN) T cells lack both CD4 and CD8 co-receptors and can be found as a rare T cell subpopulation in the periphery (1 – 5 % of CD3^{+} T cells) [69,70]. They are enriched in the bone marrow (BM), where they constitute up to one-third of

$CD3^+$ T cells [71]. While details remain elusive, the heterogenous DN T cells can be of thymic or extrathymic origin and exert proinflammatory as well as immunosuppressive effects [72]. Their potential role in immune responses, disease and therapy has increasingly gained recognition [72-75].

B cells and antibody-mediated immunity further contribute to host defense against *T. gondii* [76-84]. However, there are major differences between B cell subpopulations. B-1 cells are mainly of fetal origin and have self-renewing capacities, whereas conventional (B-2) cells need to be continuously generated from progenitors in the adult BM [85]. Most B-2 cells leave the BM at the transitional stage and complete their maturation in secondary lymphatic organs like the spleen, from where they may circulate through the blood stream and re-enter the BM as recirculating mature B cells [86,87]. While B-1 cells are a rare subpopulation in the BM, blood, spleen and lymph nodes (0.1 – 2 % of $CD19^+$ B cells), they are enriched in body cavities such as the peritoneal and pleural cavities (35 – 70 % “) [88]. They are considered innate-like B cells due to their more restricted B cell receptor repertoire, their role as major source of natural antibodies, and their capability of rapid, mainly T cell-independent secretion of low-affinity IgM during early infection [89-94]. Depending on the cell surface expression of CD5, B-1 cells are further divided into B-1a ($CD5^+$) cells, which are considered as the main natural antibody producers, and B-1b ($CD5^-$) cells, which are more involved in the adaptive antibody response than B-1a cells [91]. While B-1 cells have been shown to contribute to protection against *T. gondii* alongside conventional B-2 cells [81,84], they are also a predisposed source of autoantibodies induced by *T. gondii* infection and can negatively impact host defense [95,96]. However, the interplay between protozoan parasites and the B cell response is diverse and not fully understood [97-100].

In our previous studies, we showed that $LT\beta R^{-/-}$ mice are highly susceptible and rapidly succumb to *T. gondii* [17,26]. The expression of IFNγ and downstream effector molecules, such as mGBPs, was significantly delayed. Adaptive immunity of $LT\beta R^{-/-}$ mice was also affected, as evidenced by reduced secretion of IFNγ by $CD4^+$ T cells and cytotoxic granules by $CD8^+$ T cells after *ex vivo* stimulation [17]. B cells in the spleen of WT mice were significantly reduced during *T. gondii* infection, but this reduction was attenuated in $LT\beta R^{-/-}$ mice. Additionally, $LT\beta R^{-/-}$ mice showed only low amounts of parasite-specific IgM in the serum, and abrogated parasite-specific immunoglobulin heavy-chain class-switching and IgG production [17].

Here, we expand our investigation of the B cell immune response and B cell subpopulations to the BM and peritoneal cavity (PerC) of $LT\beta R^{-/-}$ mice and demonstrate its intriguing dynamic during *T. gondii* infection. A striking phenotype of the $LT\beta R^{-/-}$ BM was increased mature B cell frequencies, which displayed a unique resistance to inflammation-induced reduction compared to WT mature B cells. While $MHCII^+$ proinflammatory monocytes were diminished in $LT\beta R^{-/-}$ compared to WT BM, plasma cell (PC) frequencies were comparable or even increased; however, the majority of $LT\beta R^{-/-}$ BMPCs

displayed surface IgM rather than IgA, in contrast to WT BMPCs. Additionally, we found that T cell subset frequencies were altered in the BM of LTβR$^{-/-}$ mice. The mRNA sequencing of LTβR$^{-/-}$ and WT BM revealed significantly reduced interferon-related gene sets in the LTβR$^{-/-}$ BM. At the same time, the LTβR$^{-/-}$ BM exhibited a persistent upregulation of these gene sets until day 9 post-infection (p.i.), whereas the WT BM began to show their downregulation starting at day 6 p.i. In the LTβR$^{-/-}$ PerC, B-2 and B-1b cell frequencies were increased, but B-1a frequencies were comparable to WT counterparts, leading to altered B-1a to B-1b ratios in LTβR$^{-/-}$ mice. Finally, the predominant presence of neutrophils over T cells in the LTβR$^{-/-}$ PerC on day 9 p.i. marks a profound difference, showing that parasite replication is not effectively inhibited despite neutrophil accumulation, which likely contributed to exacerbated immunopathology. These results will hopefully enhance our understanding of the role of LTβR signaling in immunity during intracellular parasite infections.

Results

Mature B cells are increased in the BM of LTβR$^{-/-}$ mice and show less severe reduction during *T. gondii* infection

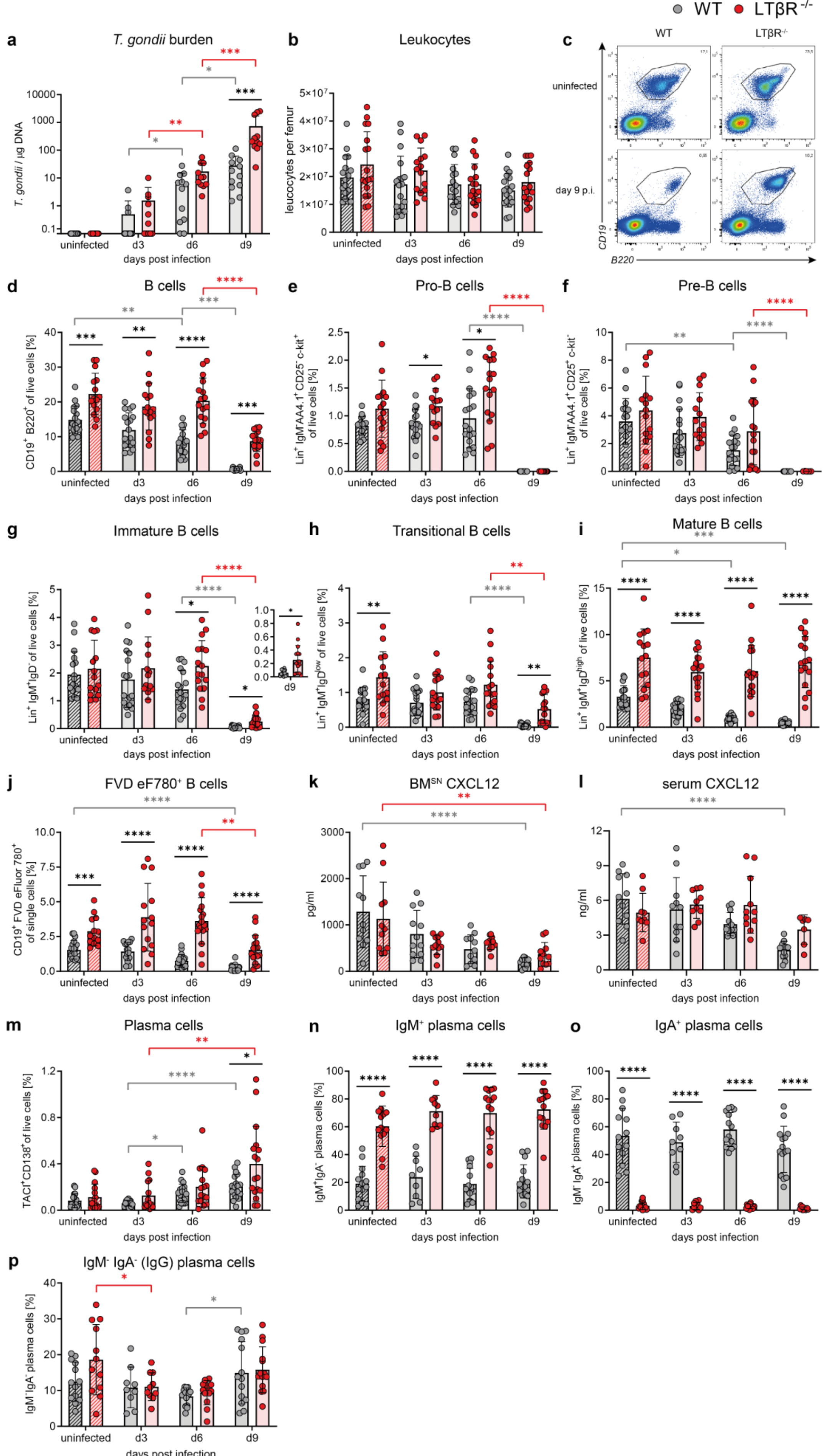
WT and LTβR$^{-/-}$ mice were intraperitoneally (i.p.) infected with *T. gondii* (ME49) and analyzed on days 3, 6 and 9 p.i. In the BM, *T. gondii* burdens increased over the course of infection; on day 9 p.i., LTβR$^{-/-}$ mice showed significantly increased *T. gondii* numbers (>20-fold) as compared to WT mice (Fig. 1a). At the same time, total leukocyte numbers per femur did not significantly differ or change between genotypes over the course of *T. gondii* infection (Fig. 1b). Interestingly, CD19^{+} B220^{+} pan-B cells were already significantly increased in LTβR$^{-/-}$ BM before infection and remained higher during infection compared to WT BM (Fig. 1c & d, S1, S2a). Pan-B cell frequencies were found significantly reduced in WT BM by day 6 p.i. but remained stable in LTβR$^{-/-}$ BM at this point (Fig. 1c & d). Both genotypes showed a significant reduction of pan-B cells from day 6 to day 9 p.i. However, the overall pan-B cell reduction was less severe in LTβR$^{-/-}$ mice (-61.0 % on day 9 p.i. compared to uninfected) compared to WT mice (-95.7 % on day 9 p.i. compared to uninfected), mirroring the course of splenic B cell frequencies after *T. gondii* infection as described previously [248].

To identify which B cell subpopulation(s) was responsible for the increased B cell numbers in the LTβR$^{-/-}$ BM, B cell developmental stages were investigated based on surface marker expression (Fig. S1, Suppl. Table 1 & 2) before and during *T. gondii* infection. Pro-, pre- and immature B cell populations were mostly comparable between genotypes (Fig. 1e – g, S2b – d), whereas transitional B cells (Lin^{+} IgM^{+} IgD^{low}, Fig. 1h, S2e) were substantially increased in uninfected LTβR$^{-/-}$ mice and on day 9 p.i.. Notably, mature B cells (Lin^{+} IgM^{+} IgD^{high}) were the primary contributors to the increased BM B cell population

in LTβR$^{-/-}$ mice, as they were significantly increased at all examined time points compared to their WT counterparts (Fig. 1i, S2f).

The strong reduction of B cells on day 9 p.i. was observed across all investigated B cell subpopulations in the WT BM (Fig. 1e – i, S2b – f). While this was also evident in early-stage B cell populations in the LTβR$^{-/-}$ BM, the transitional and mature B cells were notable exceptions. In detail, on day 9 p.i., pro-, pre-, and immature B cells were nearly absent in both WT and LTβR$^{-/-}$ BM (Fig. 1e – g, S2b – d). However, transitional B cells were significantly increased (Fig. 1h, S2e), and mature B cells remained unaffected in LTβR$^{-/-}$ BM (Fig. 1i, S2f). These results demonstrate that both phenotypes observed in the LTβR$^{-/-}$ BM – the increased B cell frequencies under steady-state conditions and the enhanced resistance to reduction during *T. gondii* infection – are specific to late-stage B cell populations, with mature B cells exhibiting the most significant alterations.

To investigate whether the reduction of B cells on day 9 p.i. was caused by B cell death or by mobilization and egress, we used a viability dye (FVD eF780) to detect dead cells (Fig. S1, Suppl. Table 1 & 2). LTβR$^{-/-}$ mice had significantly increased frequencies of dead B cells in the BM before and during *T. gondii* infection (Fig. 1j), even though both genotypes showed comparable frequencies of dead BM leukocytes (Fig. S2g). On day 9 p.i., frequencies of dead B cells were lower compared to previous days, possibly due to the overall reduced presence of B cells on that day in both genotypes (Fig. 1j). Similar to the results described for living cells, dead early (FVD eF780^{+} CD19^{+} AA4.1^{+} IgM^{-}) B cells were almost completely absent on day 9 p.i. in both WT and LTβR$^{-/-}$ animals (Fig. S2h), while dead mature B cells (FVD eF780^{+} CD19^{+} IgM^{+} IgD^{+}) were significantly increased in LTβR$^{-/-}$ animals (Fig. S2i).



WT
LTβR -/-
a
T. gondii burden
T. gondii / µg DNA
days post infection
b
Leukocytes
leucocytes per femur
c
uninfected
day 9 p.i.
CD19
B220
d
B cells
CD19+ B220+ of live cells [%]
e
Pro-B cells
f
Pre-B cells
g
Immature B cells
h
Transitional B cells
i
Mature B cells
j
FVD eF780+ B cells
k
BMSN CXCL12
pg/ml
l
serum CXCL12
ng/ml
m
Plasma cells
n
IgM+ plasma cells
o
IgA+ plasma cells
p
IgM- IgA- (IgG) plasma cells
uninfected
d3
d6
d9

Figure 1: Altered B cell subpopulations and CXCL12 concentrations in the BM of *T. gondii*-infected WT and LTβR$^{-/-}$ mice. (a) DNA was isolated from the BM and used to assess the *T. gondii* burden *via* quantitative real-time PCR of the B-1 gene (*TgB-1*). A standard curve generated from a defined number of ME49 tachyzoites (2914 ± 214/µl) was used to calculate parasite loads in WT (n ≥ 10/group) and LTβR$^{-/-}$ (n ≥ 8/group) mice. **(b)** Counted leukocytes per femur from uninfected and infected WT (n ≥ 17/group) and LTβR$^{-/-}$ (n ≥ 15/group) mice. Using surface marker staining and flow cytometry (gating strategy: Fig. S1), the following immune cell populations were identified in the BM of WT (n ≥ 15/group) and LTβR$^{-/-}$ (n ≥ 13/group) mice and quantified as percentages of live cells, unless otherwise specified: **(c & d)** Pan-B cells (CD19^{+} B-220^{+}), (c) shows a set of representative images. **(e – i)** Lin^{+} = CD19^{+} B-220^{+}. (e) Pro-B cells (Lin^{+} IgM^{-} AA4.1^{+} CD25^{-} c-kit^{+}), (f) pre-B cells (Lin^{+} IgM^{-} AA4.1^{+} CD25^{+} c-kit^{-}), (g) immature B cells (Lin^{+} IgM^{+} IgD^{-}), (h) transitional B cells (Lin^{+} IgM^{+} IgD^{low}) and (i) mature B cells (Lin^{+} IgM^{+} IgD^{high}). **(j)** Dead B cells (CD19^{+} FVD eFluor780^{+}), % of single cells. **(k & l)** CXCL12 measured in the (k) BM^{SN} and (l) serum of uninfected and infected WT (n ≥ 10/group) and LTβR$^{-/-}$ (n ≥ 7/group) mice *via* bead-based immunoassay (LegendPlex, BioLegend, USA). **(m)** Plasma cells (TACI^{+} CD138^{+}). WT: n ≥ 13/group, LTβR$^{-/-}$: n ≥ 14/group. **(n – p)** IgM^{+}, IgA^{+}, and IgM^{-} IgA^{-} (IgG) expressing plasma cells (surface Ig), % of plasma cells. WT: n ≥ 9/group, LTβR$^{-/-}$: n ≥ 10/group. All data shown represent at least four independent experiments; symbols represent individual animals and columns represent means ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

The chemokine C-X-C motif chemokine 12 (CXCL12) is, among other functions, essential for hematopoiesis and regulating B cell development by directing and retaining them in specific BM niches [101]. During inflammation, a reduction in CXCL12 has been shown to lead to the mobilization and egress of B cell precursors from the BM [102]. CXCL12 concentrations were therefore determined in serum and BM supernatant (BM^{SN}) of WT and LTβR$^{-/-}$ mice. In line with previous reports, CXCL12 concentrations were significantly reduced in WT BM^{SN} on day 9 p.i. compared to uninfected controls, a reduction which was also observed in LTβR$^{-/-}$ BM^{SN} (Fig. 1k). However, only WT mice showed a significant reduction of CXCL12 in the serum on day 9 p.i. (Fig. 1l). While the reduction of BM^{SN} CXCL12 concentrations after infection can explain the egress of early B cell populations and thus the almost complete loss of pro- and pre-B cells observed on day 9 p.i., early B cells (AA4.1^{+}) were decreased, rather than increased, in the peripheral blood (PB) on day 9 p.i. (Fig. S3a). Instead, a significantly increased AA4.1-positive myeloid (CD11b^{+}) population was identified (Fig. S3b & c). Overall, LTβR$^{-/-}$ mice showed higher numbers of circulating PB leukocytes (Fig. S3d) and increased frequencies of PB (mature) B cells (Fig. S3e & f) compared to WT controls. In contrast to the unaffected frequencies of mature B cells in the LTβR$^{-/-}$ BM, their frequency in the PB was reduced on day 9 p.i. but was still significantly higher than in WT animals (Fig. S3f).

In contrast to CXCL12, concentrations of B cell activating factor (BAFF), another cytokine important for B cell development, remained stable in the BM^{SN} (Fig. S4a) and increased in the serum (Fig. S4b) over the course of *T. gondii* infection. However, BAFF concentrations were always comparable between WT and LTβR$^{-/-}$ mice.

Surprisingly, despite lacking lymph nodes, PPs [11], and GC reactions [12,21], LTβR$^{-/-}$ mice had equal or increased (day 9 p.i.) frequencies of BMPCs (TACI^{+} CD138^{+} [103]) compared to WT mice (Fig. 1m, S4c & d), the origin of which remains unclear. However, the majority of LTβR$^{-/-}$ BMPCs were surface

IgM positive, which is in contrast to the predominantly IgA-positive WT BMPCs (Fig. 1n & o, S4f & g). Double negative (IgM^- IgA^-), presumably IgG^+, PC frequencies were comparable between genotypes (Fig. 1p, S4h).

In summary, the $LT\beta R^{-/-}$ BM contained significantly increased frequencies of mature B cells under steady-state conditions, which were also more resistant to reduction during *T. gondii* infection compared to WT counterparts. However, frequencies of earlier B cell developmental stages were comparable between genotypes, which both exhibited a significant reduction in CXCL12 concentrations in the BM^{SN} over the course of infection.

Characterization of T-, dendritic-, NK-cell and proinflammatory monocyte/macrophage populations in the $LT\beta R^{-/-}$ BM

Next, a detailed analysis of BM immune cell populations, such as T cell, DC and NK cell (sub)populations (Fig. S5 & 6, Suppl. Table 1 & 2) was conducted.

BM T cell frequencies ($CD3e^+$) were comparable between mouse genotypes and were not significantly altered during *T. gondii* infection (Fig. 2a, S7a). Interestingly, T cell subset frequencies differed significantly between both genotypes: double-negative (DN) T cells were 2-3-fold lower, while $CD4^+$ and $CD8^+$ T cells were higher in $LT\beta R^{-/-}$ BM compared to WT BM, both prior to and during *T. gondii* infection (Fig. 2b – e). These genotype-dependent differences were also partially observed in the absolute cell numbers for DN T cells and $CD4^+$ T cells but were not significant for $CD8^+$ T cells (Fig. S7b – d).

In addition to B and T cells, one of the most striking phenotypes was the reduced upregulation of surface MHCII on proinflammatory ($Ly6C^+$) monocytes/macrophages in the BM of $LT\beta R^{-/-}$ mice (Fig. 2f & g, S7e). A similar reduction was found in the PB (Fig. S7f) but not in the PerC (Fig. S7g), where MHCII-expressing proinflammatory monocyte/macrophage frequencies were comparable between genotypes. Notably, the overall frequencies of proinflammatory monocytes/macrophages (defined as $CD3e^-$ $CD19^-$ $NK1.1^-$ $CD11b^+$ $Ly6G^-$ $Ly6C^+$; gating strategy: Fig. S6) were similar between $LT\beta R^{-/-}$ and WT BM, except on day 9 p.i., where a reduction was observed in $LT\beta R^{-/-}$ BM (Fig. 2h). However, this difference was not reflected in the absolute cell numbers (Fig. S7h).

LTβR signaling is also essential for the generation of functional NK cells [12,104], NKT cells [12,14,15] as well as DCs [5,12,105]. We can confirm these findings in the BM compartment of $LT\beta R^{-/-}$ mice, where basically no NK (Fig. 2i, S7i) nor NKT cells (Fig. 2j, S7j) were detectable before and during *T. gondii* infection, and where pDCs were reduced particularly on day 6 p.i. compared to WT mice (Fig. 2k, S7k). Notably, BM neutrophil frequencies (Fig. S7l) and numbers (Fig. S7m) did not significantly differ

between genotypes. These results show that the absence of LTβR signaling perturbed specific myeloid and lymphoid immune cell populations in the BM.

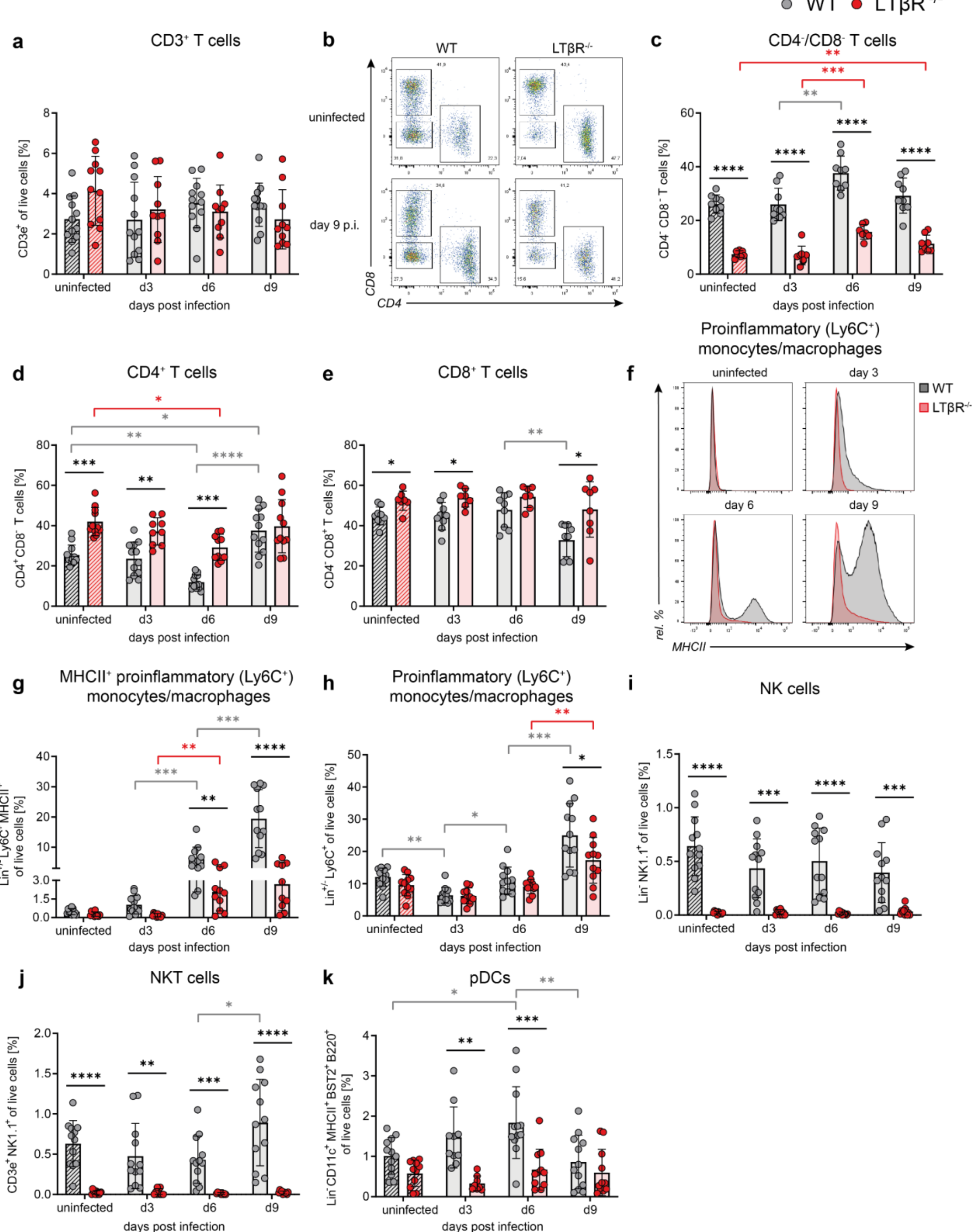


Figure 2: Altered T cells, proinflammatory monocytes/macrophages, NK cells, NKT cells and pDCs in the BM of *T. gondii*-infected LTβR$^{-/-}$ mice. Using surface marker staining and flow cytometry, the following immune cell populations (gating strategies: Fig. S5 & S6) were identified in the BM of WT and LTβR$^{-/-}$ mice and quantified as percentages of live cells, unless otherwise specified: **(a)** T cells (CD3e^{+}) **(b – e)** (b) shows a set of representative images of the gating of (c) double-negative (CD4^{-} CD8^{-}), (d) CD4^{+}, and (e) CD8^{+} T cells, all shown as % of CD3e^{+} T cells. WT: n ≥ 9/group and LTβR$^{-/-}$: n ≥ 7/group. **(f)** Representative histograms of MHCII expression (%) on

proinflammatory monocytes/macrophages. Each curve is scaled to 100 %. **(g)** MHCII-positive proinflammatory monocytes/macrophages ($CD19^-$ $CD3e^-$ $NK1.1^-$ $CD11b^+$ $Ly6G^-$ $Ly6C^+$ $MHCII^+$) **(h)** Proinflammatory monocytes/macrophages ($CD19^-$ $CD3e^-$ $NK1.1^-$ $CD11b^+$ $Ly6G^-$ $Ly6C^+$). **(i)** NK cells ($CD19^-$ $CD3e^-$ $NK1.1^+$) **(j)** NKT cells ($CD3e^+$ $NK1.1^+$) **(k)** Plasmacytoid DCs (pDCs; $CD19^-$ $CD3e^-$ $NK1.1^-$ $CD11c^+$ $CD11b^-$ $MHCII^+$ $B220^+$ $BST\text{-}2^+$). All data shown represent at least four independent experiments; symbols represent individual animals and columns represent mean values ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

LTβR$^{-/-}$ BM RNA sequencing reveals impaired upregulation of IFNγ- and IFNα-related gene sets but an enriched TNFα signaling, and an increased presence of eosinophil-associated transcripts

mRNA sequencing of WT and LTβR$^{-/-}$ BM was performed to investigate the BM transcriptome during *T. gondii* infection. Gene set enrichment analysis (GSEA) was employed to compare gene sets between genotypes (LTβR$^{-/-}$ vs WT) and across the infection timeline (d6 and d9 p.i. vs. uninfected controls, and d9 vs. d6 p.i.; Fig. 3, Fig. S9a – d & S10).

In the genotype comparison (Fig. 3a), the gene sets 'Interferon gamma response' and 'Interferon alpha response' were significantly enriched in the BM of all WT cohorts compared to LTβR$^{-/-}$ counterparts, particularly in uninfected controls and on day 6 p.i. In contrast, the 'TNFα signaling *via* NF-κB' gene set was significantly enriched in LTβR$^{-/-}$ BM at all time points examined. Other inflammation-related gene sets, such as 'Inflammatory response' and 'Reactive oxygen species pw', were enriched in WT BM or showed no significant difference between genotypes.

When comparing each infected genotype to its respective uninfected control, both WT (Fig. 3b) and LTβR$^{-/-}$ BM (Fig. 3c) exhibited proinflammatory gene set enrichment, particularly 'Interferon gamma response' and 'Interferon alpha response', on days 6 and 9 p.i. Notably, these gene sets were less enriched in WT BM on day 9 p.i. compared to day 6 p.i. (Fig. 3b), whereas LTβR$^{-/-}$ BM showed continued enrichment from day 6 to day 9 p.i. (Fig. 3c). The 'TNFα signaling via NF-κB', 'Inflammatory response', and 'Reactive oxygen species pathway' gene sets were enriched in the BM of both genotypes during *T. gondii* infection (Fig. 3b & c). A summary of additional GSEA gene sets is provided in Fig. S9d.

Thus, compared to WT BM, the LTβR$^{-/-}$ BM exhibited reduced enrichment of the critical 'Interferon gamma response' and 'Interferon alpha response' gene sets but showed greater enrichment of the 'TNFα signaling *via* NF-κB' pathway. Overall, both genotypes displayed increased expression of IFN-related gene sets over the course of *T. gondii* infection, with expression peaking in WT BM on day 6 p.i. but continuing to increase until day 9 p.i in LTβR$^{-/-}$ BM.

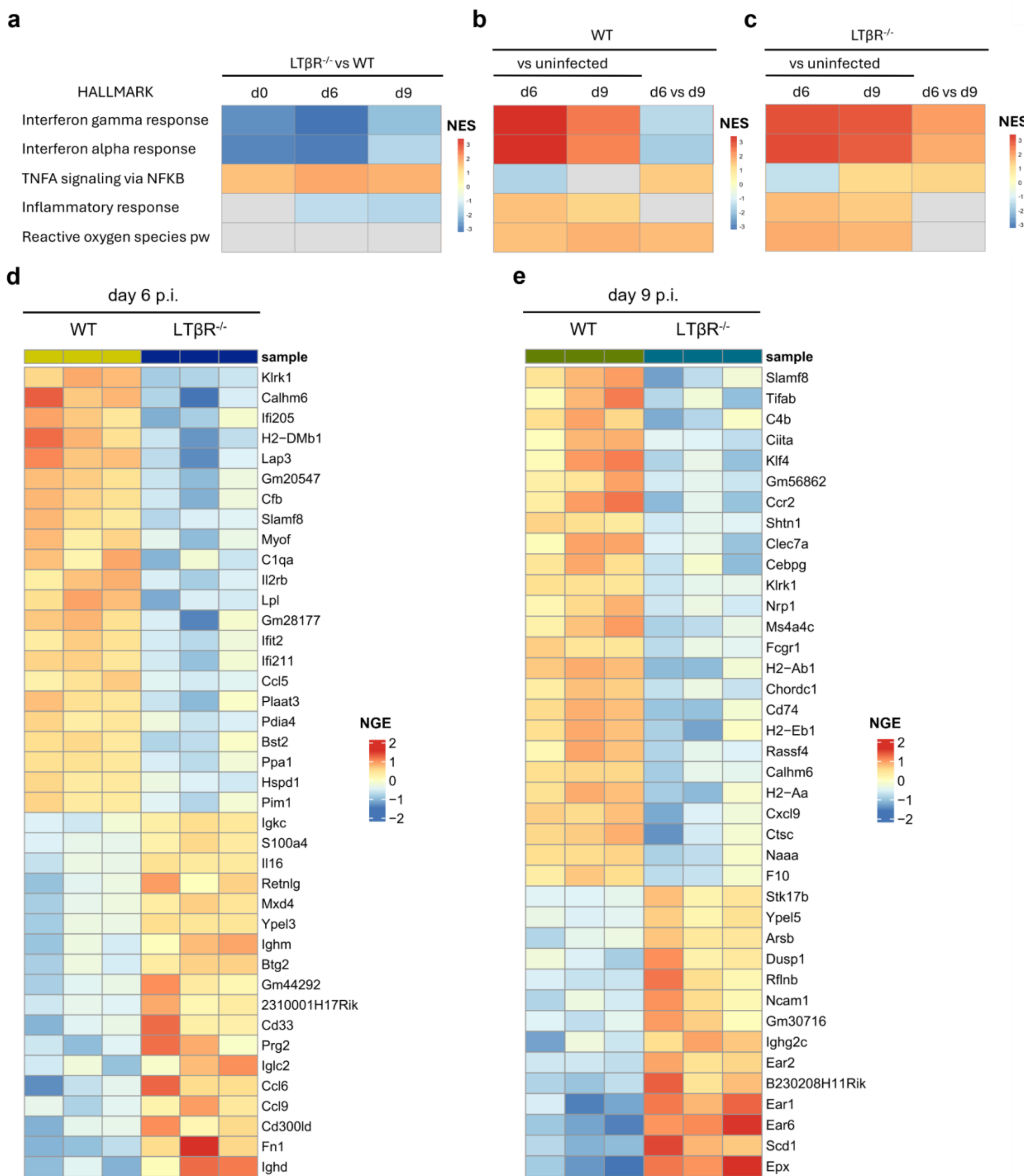


Figure 3: LTβR$^{-/-}$ BM shows less enrichment of interferon-related gene sets compared to WT BM, but more eosinophil-related gene expression on day 9 p.i. (a – c) Summarized results of the gene set enrichment analysis (GSEA) of mRNA sequencing data from BM samples of uninfected and *T. gondii*-infected WT (n = 3 /group) and LTβR$^{-/-}$ mice (n = 3, except for LTβR$^{-/-}$ uninfected (n = 2)). Normalized enrichment scores (NES) of selected GSEA hallmark gene sets (see Fig. S9a – d & S10 for complete GSEA data) are represented as colors in a heat map. A positive NES value indicates gene set enrichment in the experimental condition, a negative NES value indicates gene set enrichment in the control condition, and a gray colored tile describes non-significance (adjusted p-value > 0.01). The left panel **(a)** summarizes genotype comparisons (LTβR$^{-/-}$ vs. WT), displaying enrichment in LTβR$^{-/-}$ compared to WT controls. The middle panel **(b)** summarizes intra-WT comparisons: d6 and 9 p.i. vs. uninfected controls, displaying enrichment in infected animals; and d9 vs. d6, displaying enrichment on day 9 p.i. compared to day 6 p.i. Similarly, the right panel **(c)** summarizes intra-LTβR$^{-/-}$ comparisons: d6 and 9 p.i. vs. uninfected controls, displaying enrichment in infected animals; and d9 vs. d6, displaying enrichment on day 9 p.i. compared to day 6 p.i. Heat maps of the top 40 differentially expressed genes (based on the adjusted p-values) in WT and LTβR$^{-/-}$ BM on **(d)** day 6 p.i. and (**e**) day 9 p.i., sorted by upregulation and downregulation. NGE = normalized gene expression.

We also identified the top differential transcripts between WT and LTβR$^{-/-}$ BM on day 6 and 9 p.i. Notably, LTβR$^{-/-}$ BM displayed elevated expression of immunoglobulin genes (*Igkc*, *Ighm*, *Iglc2* and *Ighd*) and specific migration/adhesion genes (*Ccl6*, *Ccl9* and *Fn1*), but lower expression of IFNγ-inducible genes (*Ifi205*, *Ifit2*, *Ifi211* and *Bst2*) compared to WT BM on day 6 p.i. (Fig. 3d). On day 9 p.i., transcripts of several members of the eosinophil-associated, ribonuclease A family (*Ear1*, *Ear2* and *Ear6*) and *Epx* were significantly increased in LTβR$^{-/-}$ BM (Fig. 3e), whereas expression of genes associated with MHCII and antigen presentation (*Ciita*, *H2-Ab1*, *H2-Aa*, *H2-Eb1* and *CD74*), immune cell function (*Klrk1* and *Fcgr1*), and migration (*Ccr2* and *Cxcl9*) were decreased compared to WT BM (Fig. 3e). *Slamf8* transcripts, which are expressed by variety of activated myeloid cells, including IFNγ-activated monocytes and macrophages [106], were reduced on both days in LTβR$^{-/-}$ BM. Interestingly, only a small number of *T. gondii*-derived transcripts were detected, with the majority being identified in the LTβR$^{-/-}$ BM on day 9 p.i., primarily encoding GRA proteins (Fig. S9e).

Leukocyte counts, *T. gondii* burden and dead cell frequencies are elevated in the PerC of LTβR$^{-/-}$ mice

The amount of *T. gondii* tachyzoites in the PerC, as primary site of infection, was markedly increased and 10-fold higher in LTβR$^{-/-}$ mice compared to WT mice on day 9 p.i. (Fig. 4a). Both genotypes exhibited elevated peritoneal leukocyte counts compared to their respective uninfected controls. However, peritoneal leukocyte numbers were significantly higher in LTβR$^{-/-}$ mice, both before infection and on days 3 and 9 p.i. (Fig. 4b). Additionally, most, but not all, mice of both genotypes developed ascites by day 9 p.i. (data not shown). The frequency of dead cells within the total leukocyte population was found to be comparable between genotypes in uninfected controls and early during infection, but significantly higher in LTβR$^{-/-}$ mice on day 9 p.i. (Fig. 4c). Due to the higher leukocyte counts in the LTβR$^{-/-}$ PerC, the absolute number of dead cells was increased at all analyzed time points (Fig. S11a). In summary, LTβR$^{-/-}$ mice exhibited increased leukocyte counts coupled with higher parasite burden and elevated dead cells.

Frequencies of B-2 and B-1b, but not B-1a cells, are increased in the PerC of LTβR$^{-/-}$ mice

Similar to the BM phenotype, B cells in the PerC were significantly increased in LTβR$^{-/-}$ mice before and during *T. gondii* infection (Fig. 4d, S11b). The frequency of peritoneal B cells was also significantly reduced on day 9 p.i. in WT (-95.8 % on day 9 p.i. compared to uninfected) and, to a slightly lesser extent, in LTβR$^{-/-}$ mice (-90.6 % on day 9 p.i. compared to uninfected).

In addition to conventional B-2 cells (CD19^{+} B220^{high}), the PerC is enriched with B-1 cells (CD19^{+} B220^{low}), a special B cell subset with distinct origins, phenotypes, and functions [107,108]. Both B-1

and B-2 frequencies and numbers were increased in uninfected LTβR$^{-/-}$ mice (Fig. 4e – g, S11c – d). During infection, B-2 cell frequencies initially remained stable but then dropped between days 6 and 9 p.i. (Fig. 4f). In contrast, B-1 cell frequencies already dropped by day 3 p.i. and then further between days 6 and 9 p.i. (Fig 4g). Serum concentrations of CXCL13, a chemokine essential for B-1 cell functionality and mobility [109,110], increased during the course of infection and were markedly elevated on day 6 and especially day 9 p.i., but remained comparable between WT and LTβR$^{-/-}$ mice (Fig. 4h, S11e).

B-1 cells can be further classified into B-1a and B-1b cells based on their CD5 surface marker expression (S11f). The ratios between these two subpopulations were significantly altered in LTβR$^{-/-}$ mice (Fig. 4i). The LTβR$^{-/-}$ B-1a to B-1b ratio was roughly 1:1 (with slightly higher B-1b frequencies), which shifted in favor of the B-1a population over the course of infection. In contrast, WT B-1 cells mainly consisted of B-1a cells (72.0 ± 5.8 %), which slightly decreased over the course of infection. Importantly, the shift in LTβR$^{-/-}$ B-1a to B-1b frequencies was not caused by a reduction in B-1a but rather an increase in B-1b frequencies, as determined by B-1a and B-1b live cell frequencies (Fig. 4j & k). This also was observed through a direct B-1a (CD19^{+} CD5^{+}) gating without prior B-1 gating (Fig. S11g & h). Thus, B-1a cell frequencies were comparable and B-1b cell frequencies were increased in LTβR$^{-/-}$ compared to WT mice. Both B-1 subpopulations showed the two described distinct decreases (from uninfected to day 3 p.i. and from day 6 to 9 p.i., respectively). While B-1a cell frequencies were comparable between genotypes, their absolute numbers were higher in the LTβR$^{-/-}$ PerC due to the increased total leukocyte counts (Fig. 4b, S11i & j). Dead peritoneal B cells (FVD eF780^{+}) were significantly higher in LTβR$^{-/-}$ mice, particularly on day 6 and 9 p.i. (Fig. 4l, S11k).

In summary, the increased B-2 cell phenotype includes both the BM and PerC compartments in LTβR$^{-/-}$ mice. LTβR$^{-/-}$ B-1b frequencies were increased and shifted the B-1a to B-1b ratio, whereas B-1a frequencies remained comparable to the WT PerC. After *T. gondii* infection, both genotypes displayed an early reduction in B-1 cell frequencies which was absent for B-2 cells.

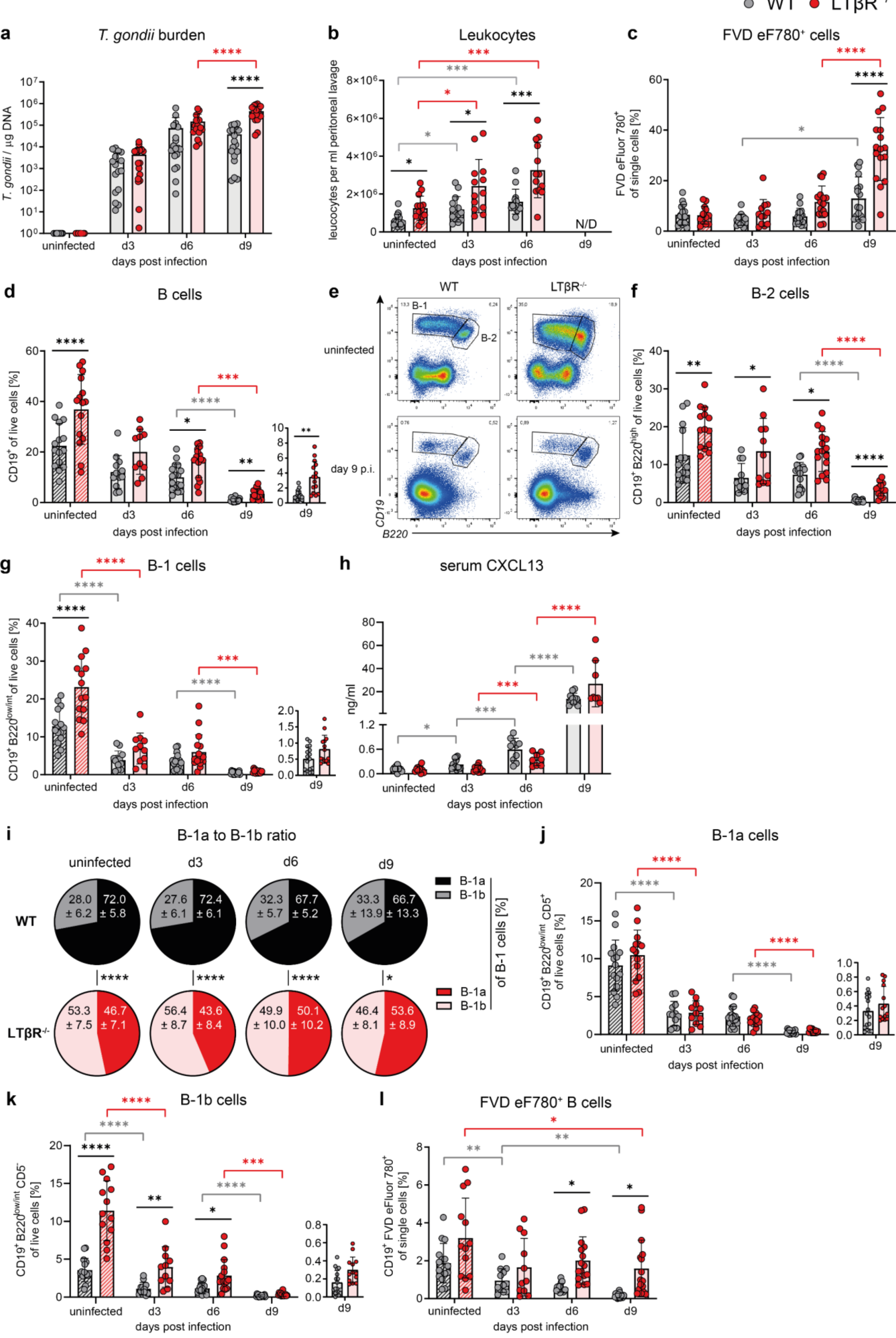

WT
LTβR-/-
a
T. gondii burden
b
Leukocytes
c
FVD eF780+ cells
d
B cells
e
f
B-2 cells
g
B-1 cells
h
serum CXCL13
i
B-1a to B-1b ratio
j
B-1a cells
k
B-1b cells
l
FVD eF780+ B cells
days post infection
uninfected
d3
d6
d9
day 9 p.i.
N/D
B-1a
B-1b
of B-1 cells [%]

Figure 4: Altered B cell subpopulations in the PerC of *T. gondii*-infected WT and LTβR$^{-/-}$ mice. (a) DNA was isolated from cells obtained by peritoneal lavage of uninfected and *T. gondii*-infected WT (n ≥ 14/group) and LTβR$^{-/-}$ (n ≥ 12/group) mice and used to assess parasite burden *via* quantitative real-time PCR of the *T. gondii* B-1 gene (*TgB-1*). A standard curve generated from a defined number of ME49 tachyzoites (2914 ± 214/µl) was used to calculate parasite loads. **(b)** Counted peritoneal leukocytes per ml lavage from uninfected and infected WT and LTβR$^{-/-}$ mice (both n ≥ 13/group). Peritoneal lavage was performed by injection of 5 ml ice-cold PBS, followed by a gentle massage and subsequent recovery of fluid. No reliable cell count can be reported for day 9 p.i. due to large amounts of cell debris and clumped cells. Exclusion of all non-single cell events during flow cytometry enabled the determination of live cell frequencies of the remaining single cells. Using surface marker staining and flow cytometry, the following immune cell populations (gating strategy: Fig. S8) were identified in the PerC of WT (n ≥ 12/group) and LTβR$^{-/-}$ (n ≥ 11/group) mice and quantified as percentages of live cells, unless otherwise specified: **(c)** dead leukocytes (FVD eFluor780^{+}), % of single cells, **(d)** B cells (CD19^{+}), and **(e – g)** B-1 (CD19^{+} B-220$^{low/int}$) and B-2 (CD19^{+} B-220^{high}) cells. (e) shows a set of representative images. **(h)** CXCL13 measured in the serum of uninfected and infected WT (n ≥ 10) and LTβR$^{-/-}$ (n ≥ 8) *via* bead-based immunoassay (LegendPlex, BioLegend, USA). **(i)** Ratios of B-1a (CD19^{+} B-220$^{low/int}$ CD5^{+}) to B-1b (CD19^{+} B-220$^{low/int}$ CD5^{-}) cells, % of B-1 cells. **(j & k)** Live cell frequencies of B-1a (CD19^{+} B-220$^{low/int}$ CD5^{+}) and B-1b (CD19^{+} B-220$^{low/int}$ CD5^{-}) cells. **(l)** Dead B cells (CD19^{+} FVD eFluor780^{+}), % of single cells. All data shown represent at least three independent experiments; symbols represent individual animals and columns represent mean values ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

The LTβR$^{-/-}$ PerC immune cell composition is dominated by neutrophils instead of T cells in late acute *T. gondii* infection

While almost absent in the PerC of uninfected controls, neutrophils increased significantly in both genotypes during *T. gondii* infection (Fig. 5a & b, S12a). By day 9 p.i., neutrophils dominated the peritoneal immune cell composition in LTβR$^{-/-}$ mice (48.4 ± 14.4 %), whereas in WT mice, T cells were the major population (42.4 ± 15.6 % pan-T cells, Fig. 5c & d). In contrast to the BM T cell phenotype (Fig. 3a – e), peritoneal T cell subset frequencies were largely comparable between genotypes (Fig. 5e – g), though CD4^{+} T cells were increased in uninfected LTβR$^{-/-}$ mice and on day 3 p.i., while DN T cells were markedly higher in WT mice on day 6 p.i. While CD8^{+} T cell frequencies were comparable between genotypes in the BM of uninfected animals, they were significantly reduced in the PerC of uninfected LTβR$^{-/-}$ mice, indicating an altered peritoneal T cell subset composition even before infection. Regarding absolute numbers, DN T cells differed significantly between genotypes in uninfected mice and on day 6 p.i., while CD4^{+} T cells and CD8^{+} T cell numbers showed no significant difference (Fig. S12b – e).

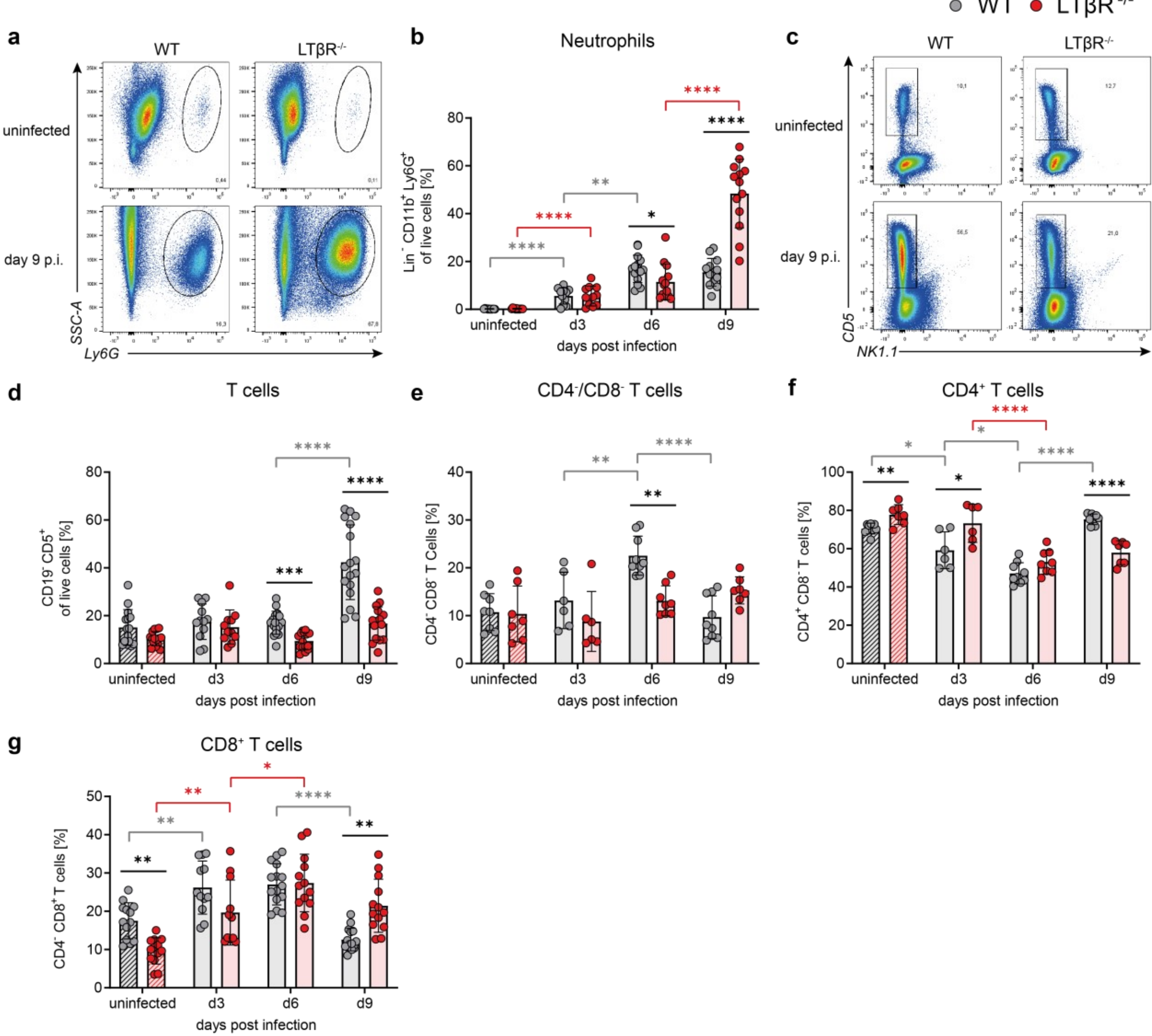


Figure 5: Neutrophils, instead of CD4+ T cells, dominate the PerC immune cell composition of LTβR-/- mice on day 9 p.i. during *T. gondii* infection. Using surface marker staining and flow cytometry, the following immune cell populations (gating strategy: Fig. S8) were identified in the PerC of WT (n ≥ 12/group) and LTβR-/- (n ≥ 11/group) mice and quantified as percentages of live cells, unless otherwise specified: **(a & b)** neutrophils (CD19- CD5- NK1.1- CD11b+ Ly6G+) (a) shows a set of representative images. **(c & d)** T cells (CD19- CD5+) (c) shows a set of representative images. **(e – g)** Double-negative (CD4- CD8-), CD4+ and CD8+ T cells, % of T cells. WT: n ≥ 6/group, LTβR-/-: n ≥ 6/group. All data shown represent at least two independent experiments; symbols represent individual animals and columns represent mean values ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

LTβR-/- mice display increased inflammatory infiltrates in lung and liver

A necropsy of lung, liver, brain, kidney, spleen and BM followed by histological analysis was performed to further investigate the impact of LTβR deficiency during *T. gondii* infection.

LTβR-/- mice are known to exhibit abnormal lymphocytic infiltrations in the lungs even in the absence of infection [11,12], a finding also evident in our histological analysis (Fig. 6). While *T. gondii* infection generally increased lung infiltration compared to uninfected controls, these inflammatory infiltrates

were more pronounced in LTβR$^{-/-}$ lungs compared to WT lungs, consistent with the phenotype observed under steady-state conditions. During *T. gondii* infection, WT animals showed minimal pulmonary pathology, characterized by mild, scattered monocytic infiltration on day 9 p.i. In contrast, LTβR$^{-/-}$ lungs displayed increased perivascular and pleural mixed inflammatory infiltrates on day 6 and 9 p.i. (Fig. 6).

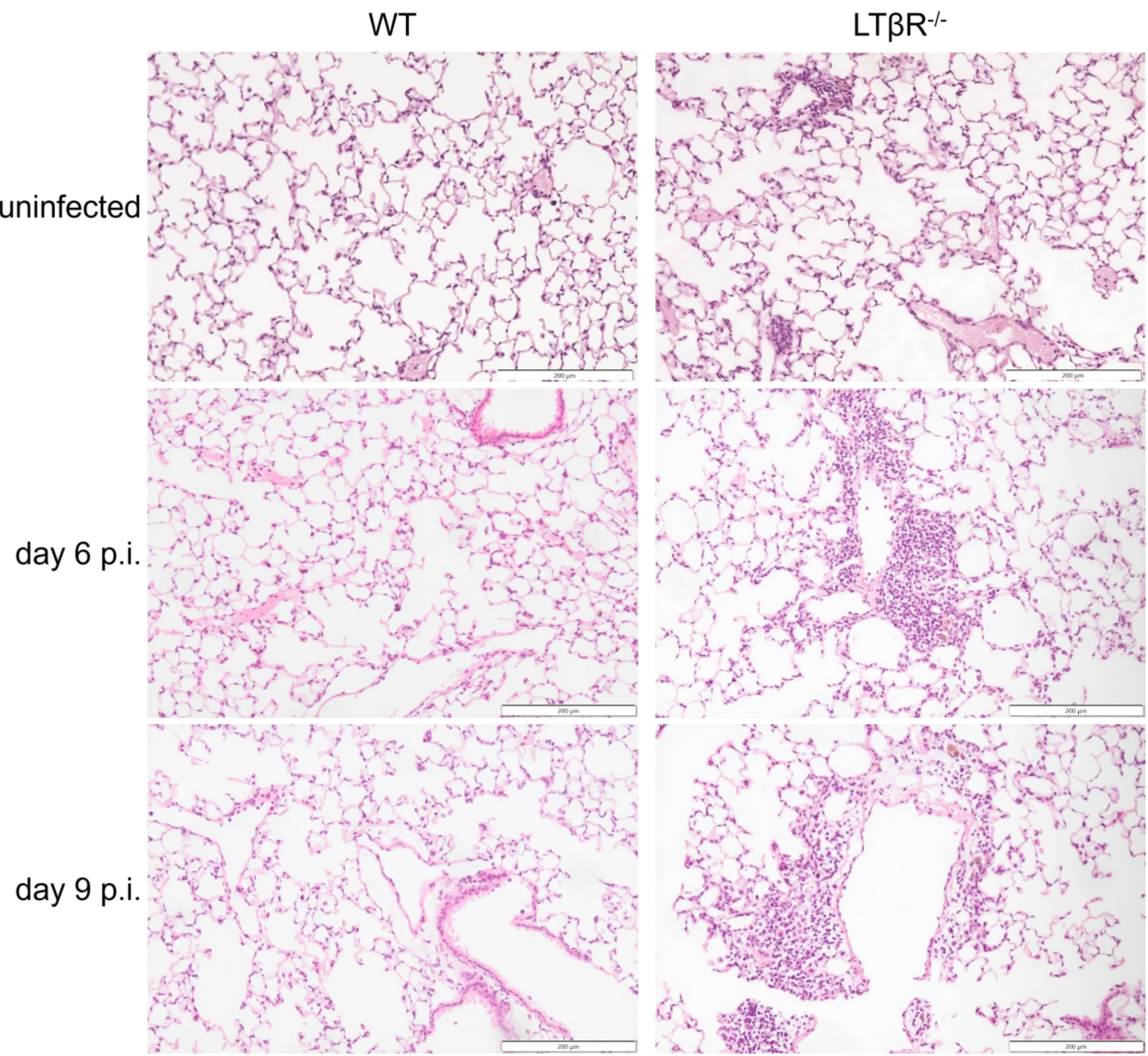


Figure 6: LTβR$^{-/-}$ mice exhibit more inflammatory infiltrations in the lung on day 6 and 9 p.i. during *T. gondii* infection. Representative H&E images of lungs from WT and LTβR$^{-/-}$ mice before and during *T. gondii* infection. n = 3 except for LTβR$^{-/-}$ uninfected (n = 1). Scale bars: 200 µm.

Overall, the liver of the uninfected LTβR$^{-/-}$ control was comparable to that of uninfected WT mice and did not exhibit pronounced lymphocytic infiltrations under steady-state conditions (Fig. 7). During *T. gondii* infection, WT mice displayed multifocal inflammatory foci occasionally associated with necrotic cells, as well as marked capsular and minimal perivascular/periportal inflammation. On day 6 p.i., these inflammatory/necrotic foci were few, scattered, and dominated by neutrophils and

cellular debris (acute necrosis); at day 9 p.i., foci were more numerous, with often macrophages predominating (subacute necrosis). In contrast, the livers of LTβR$^{-/-}$ mice displayed more extensive inflammation, with predominantly (sub)capsular, perivascular, and mixed mononuclear infiltrates, and randomly scattered areas of multiple hepatocellular necrosis and neutrophilic inflammation. Large numbers of monocytes were usually visible within the lumen of large-caliber vessels (monocytic leukocytosis). At day 6 p.i., inflammatory foci were few and <50% of vessels were affected. At day 9 p.i., most vessels had perivascular mixed mononuclear inflammation, parenchymal foci were increased in number, and macrophages predominated (subacute necrosis).

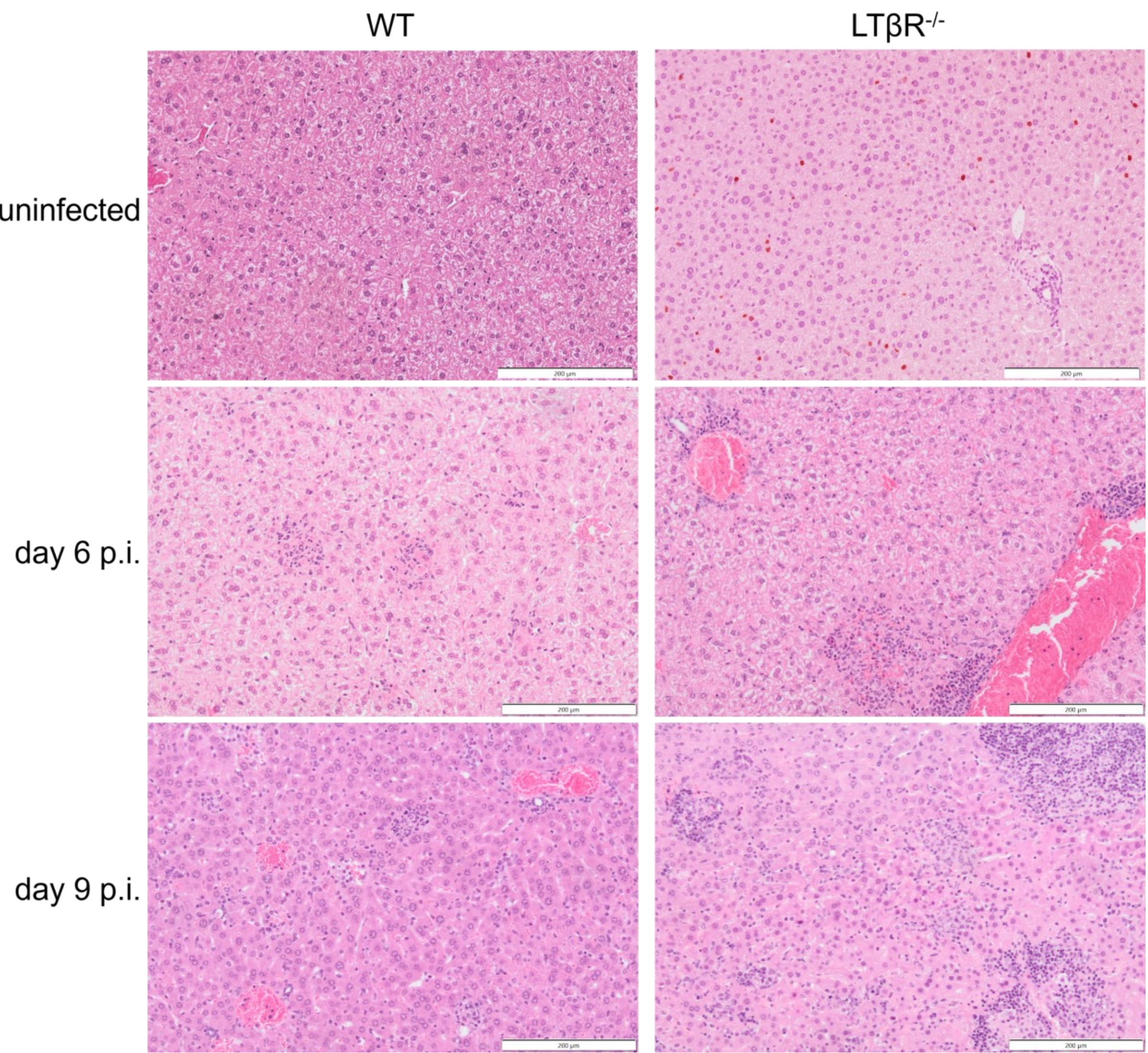


Figure 7: LTβR$^{-/-}$ mice exhibit more inflammatory infiltrations in the liver on day 6 and 9 p.i. during *T. gondii* infection. Representative H&E images of the liver from WT and LTβR$^{-/-}$ mice before and during *T. gondii* infection. n = 3 except for LTβR$^{-/-}$ uninfected (n = 1). Scale bars: 200 µm.

Neither the brain nor BM displayed genotype-specific differences in the histological analysis (data not shown). WT and LTβR$^{-/-}$ BM had mild to moderately increased granulopoiesis on day 6 and 9 p.i., as

evident by increased ratios of myeloid to erythroid cells (data not shown). The kidneys displayed mild to moderate capsular inflammatory infiltrations, but no genotype-specific differences (Suppl. Fig. 13c).

Discussion

B cells play a crucial role in both protective and, counterintuitively, sometimes in disease aggravating responses during protozoan parasite infections [78,82,95,96]. LTβR$^{-/-}$ mice, which exhibit high susceptibility to *T. gondii* infection, show a compromised ability in generating parasite-specific IgM and IgG responses [17]. In this study, LTβR$^{-/-}$ mice were investigated to further elucidate the impact of absent LTβR signaling on B cell populations in the BM and PerC during *T. gondii* infection.

On day 9 p.i., both WT and LTβR$^{-/-}$ mice exhibited reduced numbers of B cells at all identified developmental stages (pro-, pre-, and immature B cells) in the BM, as well as in the transitional B cell stage. A similar phenotype has been described during infection with other protozoa, such as *Trypanosoma brucei* [111] and *Plasmodium chabaudi* [112]. Bockstal et al. reported severe reduction in B cell developmental stages, from common lymphoid progenitors (CLP) to immature B cells, in the BM of *T. brucei*-infected mice; however, stages before CLP were only minimally affected [111]. They also noted trypanosomiasis-induced apoptosis of transitional B cells in the spleen but found no significant increase in apoptosis in early BM B cell stages. Consistent with this, we did not observe a notable rise in dead BM B cells during *T. gondii* infection. However, rapid clearance of dead cells from the BM has been reported [113,114], which suggests the need for a different approach for accurate quantification, also considering efferocytosis may play a role in the developmental stage B cell reductions. Generally, *T. gondii* can modulate host-cell apoptosis to persist in host cells. In contrast, *T. gondii* can also initiate apoptosis in some cell types during specific stages of infection [115]. In contrast to WT mice, transitional B cell and mature B cell frequencies remain high in infected LTβR$^{-/-}$ BM, indicating a role for LTβR signaling for the reduction of these B cell differentiation stages in the BM.

Rather than apoptosis and/or cell death, mobilization and egress caused by reduced stromal CXCL12 expression seem to drive the reduction of early B cells in the BM during *T. brucei* [111] and *P. chabaudi* [112] infection. A comparable mechanism appears to occur in *T. gondii* infection (this study). The chemokine CXCL12 is crucial for B cell development and retention in the BM [116-118]. During inflammation, proinflammatory cytokines, especially TNFα, downregulate stromal CXCL12 expression, leading to early B cell mobilization and impaired lymphopoiesis [102]. However, we did not find a significant increase in early B cells in the blood at day 9 p.i., and their exceptional sensitivity to

apoptosis [119] suggests that most mobilized early B cells might have died and been cleared from the blood by this time.

Mature B cells were the major subset responsible for the increased BM B cell frequencies observed in LTβR$^{-/-}$ mice. Despite a significantly higher parasite burden and reduced CXCL12 concentrations, mature B cell frequencies remained unaffected by *T. gondii*-infection in LTβR$^{-/-}$ BM, whereas these stages decreased over the course of infection in WT BM. This result raises two questions: i) What caused the increased frequency of mature B cells in the BM of uninfected LTβR$^{-/-}$ mice? ii) why were the mature B cell frequencies not reduced in LTβR$^{-/-}$ BM during *T. gondii* infection, in contrast to WT BM? Here, several factors and interactions deserve consideration:

ad i) Altered migration: in uninfected LTβR$^{-/-}$ mice, B-2 cell frequencies were increased not only in BM but also in the PerC (this study) and in the spleen [17]. Frequencies of early B cell stages (pro-, pre-, and immature B cells) were mostly comparable between LTβR$^{-/-}$ and WT BM, suggesting that early B cell development is not impaired in the absence of LTβR signaling. Instead, later B cell stages capable of BM egress (transitional B cells) or re-entry (mature B cells) were markedly increased in the LTβR$^{-/-}$ BM. The BM, like other SLOs, has distinct B cell areas, with mature B cells organized in extravascular perisinusoidal niches containing T cells, monocytes, and BMDCs, which these B cells can freely leave and re-enter [120,121]. Alterations in these niches or the mature B cell migration could potentially occur in LTβR$^{-/-}$ BM. This might be in analogy for the significant role of LTβR signaling in thymic T cell development, where LTβR signaling controls the homing of lymphoid precursor cells (LPC) and the egress of mature thymocytes [18]. It is conceivable that LTβR signaling could similarly influence B cell migratory properties within the BM.

ad ii) Role of LTβR signaling for leukocyte migration. The LTβR is expressed on endothelial cells [122] and induces synthesis of adhesion and migration molecules such as vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), E-selectin, CXCL12 and mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) [10,22,123-125]. While our BM transcriptome data did not show significant alterations in the expression of these molecules between genotypes or during *T. gondii* infection, we observed differential expression of other leukocyte migration-regulating molecules in LTβR$^{-/-}$ BM compared to WT BM. These include the C-C motif chemokine ligands 5, 6, and 9 (CCL5, CCL6, CCL9), Fibronectin 1 (FN1), C-C motif chemokine receptor 2 (CCR2), and C-X-C motif chemokine ligand 9 (CXCL9). These differences may reflect LTβR-dependent alterations in leukocyte migration or variations in the infection and immune response between LTβR$^{-/-}$ and WT mice at the time points analyzed. Also, the absence of SLOs in LTβR$^{-/-}$ mice, including lymph nodes and gut-associated lymphatic tissue (GALT) [11], may contribute to the accumulation of mature B cells in other compartments with suitable microenvironments, such as the BM and the PerC. Additionally, LTβR$^{-/-}$

mice have increased leukocyte counts in the circulation and tissue infiltrations under steady-state conditions, primarily involving $CD4^+$ T cells and B cells [11], which may relate to potentially altered migration mechanisms in the absence of LTβR signaling, as discussed earlier. Our necropsy findings corroborate these results, revealing moderate to marked perivascular inflammatory infiltrates in the lungs and livers of $LTβR^{-/-}$ mice during *T. gondii* infection which were absent or less pronounced in WT animals, indicating altered lymphocyte migration or infection dynamics. Capsular inflammation in abdominal organs, such as the kidneys in WT and $LTβR^{-/-}$ mice, likely reflects the intraperitoneal infection with *T. gondii*.

CXCL12-induced adhesion gradually diminishes over the course of B cell development, likely to enable later stages to exit the BM and complete their development in SLOs. *In vitro,* mature BM and especially blood B cells show significantly reduced adhesion responses to sustained CXCL12 stimulation compared to earlier B cell subsets [117]. Blocking CXCL12 signaling *via* CXCR4 depletion in $CD19^+$ BM B cells leads to increased B cell localization in BM sinusoids instead of the parenchyma, and significantly reduces mature B cells numbers in the BM [126]. However, this reduction might be partly due to disruptions in macrophage migration inhibitory factor (MIF) signaling, as MIF is a critical survival factor for mature BM B cells [127,128] acting through CXCR4 (and CXCR2) [129,130]. Homing of transferred B cells to the BM is also impaired in mice with reduced CXCL12 expression [102]. In conclusion, while the reduction of early and mature B cells in WT BM due to reduced levels of CXCL12 during inflammation is in line with literature, surprisingly, $LTβR^{-/-}$ BM mature B cells were unaffected by the CXCL12 reduction, suggesting retention by altered or unknown mechanisms. Therefore, the regulation of other adhesion molecules such as VCAM-1 and cannabinoid receptor 2 (CB2) [126], and migration factors such as MIF should be analyzed in the future.

Protozoan parasites can alter B cell compartments during infection. Mechanisms reported include induction of apoptosis [11, 96], polyclonal B cell activation [7, 16, 24], and autoantibody secretion [20, 21]. Therefore, increased BM mature B cells in $LTβR^{-/-}$ might have been due to increased parasite numbers in the BM compartment. Taken together, the (combined) mechanisms discussed above could be responsible for the increased mature B cell numbers in $LTβR^{-/-}$ BM, which displayed increased reduction-resistance compared to WT counterparts during *T. gondii* infection.

BMPC ($TACI^+$ $CD138^+$) frequencies increased over the course of *T. gondii* infection and were significantly higher on day 9 p.i. in $LTβR^{-/-}$ BM compared to WT BM. This was surprising considering the lack of lymph nodes and GALT, as well as the dysfunctional splenic GC formation observed in $LTβR^{-/-}$ mice [11,12,21]. One possibility is alternative PC differentiation in the BM, which can occur independently of GC B cell maturation or T cells [120,131,132]. Additionally, B-1 cell derived BMPCs ($CD138^+$), which are a major source of natural IgM [92,108], might constitute the observed population of IgM^+ PCs in the $LTβR^{-/-}$

BM. The severe reduction of IgA^+ BMPCs reported here is in concordance with previous reports of reduced IgA production in $LT\beta R^{-/-}$ mice [12,133]. In the gut, PPs, mesenteric lymph nodes (MLNs), and the lamina propria (LP) are primary compartments for IgA production; however, PPs and MLNs are absent in $LT\beta R^{-/-}$ mice [11], and the LP is dependent on stromal LTβR expression for B cell migration [133]. Overall, LTβR signaling seems to be crucial for IgA^+ PC generation, prior to and after pathogen infection.

In addition to B cells, T cells were also altered in the BM of $LT\beta R^{-/-}$ mice. In contrast to BM B cells, pan-T cell ($CD3e^+$) frequencies were not substantially increased in $LT\beta R^{-/-}$ BM, despite their shared ability to express LTβR-ligands, their overlapping BM niches [121], and their similarly increased presence in the PB and organ infiltrations in $LT\beta R^{-/-}$ mice [11]. Furthermore, BM T cells were not significantly reduced over the course of *T. gondii* infection, which was surprising because T cells, like B cells, are reduced in presence of proinflammatory cytokines [102]. While pan-T cells were comparable between genotypes, we found T cell subset frequencies to be significantly shifted in $LT\beta R^{-/-}$ BM, showing decreased DN T cell and increased $CD4^+$ and $CD8^+$ T cell frequencies compared to the WT BM. The ontogeny of DN T cells remains elusive, with evidence supporting both thymus-dependent and thymus-independent pathways, including differentiation from both $CD4^+$ and $CD8^+$ T cells [72]. *In vitro*, it was shown that immature BMDCs can drive regulatory DN T cell development from non-regulatory $CD4^+$ T cells, and that these induced DN T cells interfere with B cell proliferation and differentiation into PCs, promoting B cell apoptosis [134]. Absent LTβR signaling in BMDCs could impair this pathway and lead to the shifted $CD4^+$ and DN T cell frequencies observed in $LT\beta R^{-/-}$ BM. Alternatively, it is possible that thymic DN T cell development pathways are impaired, as LTβR signaling plays a role during T cell development in the thymus [18].

A striking phenotype in the $LT\beta R^{-/-}$ BM was the reduced upregulation of MHCII expression on the surface of proinflammatory ($Ly6C^+$) monocytes/macrophages as compared to their WT counterparts. This observation is also reflected by the BM transcriptome data, which shows significantly lower expression of multiple genes related to MHCII and antigen presentation in $LT\beta R^{-/-}$ BM compared to WT BM. It is known that prior to BM egress, $Ly6C^+$ monocytes and their precursors are primed with IFNγ produced by BM-resident NK cells during infection, a process that includes the upregulation of MHCII expression [135,136]. BM NK cells themselves are activated in response to IL-12 originating from *T. gondii*-infected cells. Our data revealed a marked reduction, but not a complete absence, of $MHCII^+$ $Ly6C^+$ monocytes/macrophages in the $LT\beta R^{-/-}$ BM during *T. gondii* infection. This reduced presence of $MHCII^+$ $Ly6C^+$ monocytes/macrophages is likely due to the lower levels of IFNγ, given that NK cells are severely reduced in $LT\beta R^{-/-}$ mice [12,16], including, as shown here, the BM. This is further supported by a significantly delayed systemic increase of IFNγ in $LT\beta R^{-/-}$ mice compared to WT mice during

T. gondii infection [17,26], which was also detected in the transcriptome data from the BM compartment in this study.

Overall, the BM transcriptome data indicate that although the immune response, particularly IFN-related genes, is upregulated during *T. gondii* infection in LTβR$^{-/-}$ BM, it remains markedly reduced compared to the WT BM. Interestingly, the increase in eosinophil-associated transcripts in LTβR$^{-/-}$ BM on day 9 p.i., including *Ear1, Ear2, Ear6,* and *Epx* – transcripts that are characteristic of eosinophil precursors and immature eosinophils during development [137] – may be linked to this reduced and delayed IFNγ response, as IFNγ is known to inhibit eosinophil differentiation [138,139]. Furthermore, while IFN-related gene set enrichment persisted in LTβR$^{-/-}$ BM throughout the infection, its decline from day 6 to day 9 p.i. in WT BM suggests immune downregulation, likely reflecting emerging parasite control and the need to prevent excessive immunopathology. Additionally, the 'TNFα signaling *via* NF-κB' gene set was profoundly enriched in LTβR$^{-/-}$ BM at all investigated time points. As this enrichment was already present in uninfected mice, it does not appear to be driven by *T. gondii* infection but may result from compensatory mechanisms attempting to "balance" the missing LTβR signals [140]. The LTβR and TNF receptors (TNFR1/TNFRp55 and TNFR2/TNFRp75) share downstream signaling components of the classical NF-κB pathway [141-143], which could explain this observed enrichment in the absence of LTβR signaling.

Within this study, we investigated the PerC in order to analyze the immune cell populations after *T. gondii* infection and in the absence of LTβR signaling. The PerC B-2 cell phenotype in LTβR$^{-/-}$ mice closely mirrors that of the BM, with significantly elevated frequencies compared to the WT, both before and during infection, and particularly on day 9 p.i. Alterations in the local microenvironment, migration, and/or signaling, as discussed for BM B cells, should be considered as potential contributing factors here, too. Additionally, PerC B-1 cells displayed an early reduction by day 3 p.i. in both genotypes following *T. gondii* infection, a change not observed in B-2 cells.

The PerC is a compartment enriched with B-1 cells. In previous reports, peritoneal B-1 cell reduction has been reported after i.p. infection, with kinetics ranging from 3 hours (following *Escherichia coli* injection, associated with increased B-1 cell egress [110]) to 15 days (after *Trypanosoma cruzi* infection, associated with B-1 cell differentiation into peritoneal plasma cells rather than increased apoptosis [144]). After *T. gondii* infection, we also observed an early reduction of B-1 cells by day 3 p.i., independent of LTβR signals. We hypothesize that B-1 cell egress, with kinetics specific to an intraperitoneal *T. gondii* infection, accounts for this decline.

In our previous study, a host-pathogen network prediction model, based on RNA-seq data from murine lung tissue after *T. gondii* infection, suggested that the loss of LTβR signaling could lead to the increased expression of a gene set containing 516 genes, with the top gene ontology (GO) term identified as the

"B cell receptor signaling pathway" [17]. Assuming that altered BCR signaling contributes to the observed B cell phenotype in LTβR$^{-/-}$ mice, the relative unresponsiveness of peritoneal B-1 cells [88,145-147] – particularly B-1a cells [148,149] – to BCR stimulation, compared to B-2 cells, may explain why B-1a cells were exempt from the otherwise increased B cell populations in LTβR$^{-/-}$ mice.

Furthermore, the domination of the PerC immune cell composition by neutrophils in LTβR$^{-/-}$ mice on day 9 p.i. marked another significant alteration compared to the WT PerC, which was dominated by CD4^{+} T cells at this time. These increased amounts of neutrophils, along with a higher number of dead cells, may indicate exacerbated peritoneal inflammation and potential immune pathogenesis in LTβR$^{-/-}$ mice. This finding also suggests that the increased accumulation of neutrophils was not sufficient to effectively control parasite replication in the PerC. Although we did not specifically assess neutrophil functionality in LTβR$^{-/-}$ mice, it is noteworthy that LTβR signaling has been shown to prevent otherwise exacerbated dextran sodium sulfate (DSS)-induced colitis in mice by suppressing energy-metabolic pathways in neutrophils [3]. Therefore, the absence of LTβR signaling might further exacerbate immunopathology caused by accumulated neutrophils in the LTβR$^{-/-}$ PerC. Also, it might be speculated that LTβR signals are required to fully activate neutrophils for control of *T. gondii* replication.

The T cell subset alterations were less pronounced in the LTβR$^{-/-}$ PerC compared to the BM, with the most significant changes being a reduced CD8^{+} T cell frequency in uninfected controls and a reduced CD4^{+} T cell frequency compared on day 9 p.i. compared to the WT PerC. The CD4^{+} T cell subset potentially includes IFNγ-producing T helper type 1 (Th1) cells, which play a critical yet double-edged role during *T. gondii* infection [43,63,65,150], as well as regulatory T cells (T_{regs}), which limit immunopathology and are more dependent on LTβR signaling for migration than other T cell subsets [66,151,152].

In conclusion, our study identifies distinct and novel alterations in immune cell (sub)populations in LTβR$^{-/-}$ mice prior to and during *T. gondii* infection. This includes elevated frequencies of mature B cells in the BM and PerC, as well as elevated frequencies of DN T cells in the BM. While the underlying mechanisms are yet to be fully elucidated, these findings suggest potential new pathways for further research that could uncover the specific roles of LTβR signaling in immune cell regulation. Additionally, our study provides valuable insights into the BM transcriptome in the absence of LTβR expression during protozoan parasite infection, thereby contributing to a deeper understanding of the complex and multifaceted roles of LTβR signaling in immune responses.

Material and Methods

Mice. LTβR$^{-/-}$ mice were described previously [11] and back-crossed to a C57BL6/N background for at least 10 generations. Wildtype (WT) littermates were used as controls. Mice were housed in the animal facility of the Heinrich Heine University Düsseldorf under specific-pathogen-free (SPF) conditions and were 8 to 16 weeks old at the time of infection. CD1 mice from Charles River Breeding Laboratories were used to maintain and propagate ME49 *Toxoplasma gondii* for infection experiments. All animal experiments were conducted in strict accordance with the German Animal Welfare Act. The protocols were approved by the local authorities (Permit no. 81-02.04.2018.A406, 81-02.04.2021.A060 and 81-02.05.40.18.082). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

T. gondii **cyst preparation and infection experiments.** ME49 (type II strain) cysts were isolated from the brain of CD1 mice 11 to 19 weeks after infection *via* Ficoll-Paque gradient centrifugation. Briefly, the murine cerebrum was homogenized by passaging through successively thinner cannulas (smallest size: 23G). After centrifugation (130 x g, 5 min, room temperature (RT)), the pellet was resuspended in 20 ml PBS. 10 ml of Ficoll-Paque Plus (GE Healthcare, USA) was carefully layered below the PBS, followed by centrifugation (1,250 x g, 25 min, RT) without brakes. Pelleted cysts were washed with PBS, counted (10 cysts per mouse) and lysed with Trypsin-EDTA. Lysis was stopped by adding heat-inactivated (56 °C, 30 min) fetal calf serum (FCS, Pan Biotech, Germany). After a final wash with PBS and centrifugation (660 x g, 10 min, RT), ME49 bradyzoites were resuspended in 0.2 ml PBS per murine recipient. Mice were infected via intraperitoneal injection and weighed and scored daily for the duration of the experiment.

Mouse material preparation and processing. On day 3, 6 and 9 p.i., mice were anesthetized with 100 mg/kg Ketamine (Zoetis, USA) and 10 mg/kg Xylazine (Elanco, USA). **Peritoneal lavage**: was performed by injection of 5 ml ice-cold PBS (22G) into the peritoneal cavity, followed by a gentle massage and extraction. Aliquots were prepared for flow cytometry and quantitative real-time PCR (qRT-PCR). **Blood/serum:** mice were bled through the *vena cava inferior* (20G). For flow cytometry, 200 µl of blood were immediately added to 50 µl 0.5 M EDTA and stored on ice. After washing with 25 ml PBS (+2% FCS, v/v) and centrifugation (470 x g, 7 min, 4 °C), red blood cells (RBC) were lysed in Erylysis-buffer (Morphisto, Germany). Leukocytes were then counted and used for flow cytometry surface staining. The remaining blood was allowed to coagulate for 30 min at room temperature (RT) followed by two centrifugation steps (10 min, 8,000 x g) to generate serum. **Bone marrow:** both murine femora were dissected, and BM cells were isolated and pooled *via* centrifugation. Briefly, femora were cut open at joint-side and placed in punctured 0.5 ml tubes that were then placed in 1.5 ml tubes. After centrifugation (12,100 x g, 15 sec, RT), BM supernatant was removed for cytokine measurement. After

RBC lysis, remaining leukocytes were counted, and aliquots were prepared for flow cytometry, qRT-PCR and RNA Sequencing.

Cell surface staining for flow cytometry. Cells were stained in U-bottom polystyrene plates and were incubated with Fc-blocking CD16/32 antibody (1:100) in staining buffer (1x PBS with 2 mM EDTA and 2% FCS (v/v)) for 30 min at 4 °C. Fluorophore-labeled antibodies were prepared in staining buffer, added to cells (final volume: 50 µl) and incubated for 30 min (4 °C, light-protected). Cells were then washed, preserved by the addition of 4% paraformaldehyde for 15 min at RT and analyzed with a BD LSRFortessa II flow cytometer and FlowJo v10.8 software. All antibodies and dyes are listed in Supplementary Table 1, all identified immune cell populations are listed in Supplementary Table 2, and all gating strategies are shown in Fig. S1, S5, S6, and S8.

Detection of parasite burden. Bone marrow and peritoneal lavage samples were centrifuged (10,000 x g, 5 min, RT), total DNA was isolated from pellets using a DNA isolation kit (Genekam, Germany) according to manual, and samples were set to 100 ng/µl DNA. TgB1 primer (Forward: 5'-GCT AAA GGC GTC ATT GCT GTT-3', Reverse: 5'-GGC GGA ACC AAC GGA AAT-3') and a FAM-probe (5'-FAM-ATC GCA ACG GAG TTC TTC CCA GAC GT-BHQ1-3') were purchased from Metabion (Germany) to amplify and detect a defined sequence of the 35-fold repetitive B1 gene from *T. gondii*. qRT-PCR was performed on Bio-Rad CFX-96 systems. DNA isolated from a defined number of ME49 tachyzoites (2.914 ± 214/µl) was used to generate a standard curve and calculate parasite loads.

Cytokine measurement. Murine cytokines BAFF, CXCL12 and CXCL13 were measured with a custom selected bead-based multiplex panel (LEGENDplex from BioLegend, USA). BM^{SN} was used undiluted, serum was processed according to manual. Samples were acquired with a BD FACSCanto II or LSRFortessa II and analyzed with Qognit software (BioLegend).

Histology. Liver, lung, spleen, kidney, brain and femora were harvested from WT and $LT\beta R^{-/-}$ mice, fixed in 4 % neutral buffered formalin for 48 hours, and stored in 70 % ethanol at 4°C until further processing. 5 µm sections were cut, transferred onto glass slides, and stained with a standard hematoxylin/eosin protocol. Femur samples were decalcified in RDO solution (Apex Engineering, USA) for 1 hour before processing.

Generation of QuantSeq 3' mRNA-sequencing data. Bone marrow samples of uninfected (d0) and *T. gondii*-infected (ME49, bradyzoites from 10 cysts, i.p.) WT and $LT\beta R^{-/-}$ mice were generated as described and stabilized in RNAprotect Tissue Reagent (Qiagen, Germany). Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Germany), quantified (Qubit RNA HS Assay, Thermo Fisher Scientific, USA) and quality measured by capillary electrophoresis using the Fragment Analyzer and the 'Total RNA Standard Sensitivity Assay' (Agilent Technologies, USA). All samples in this study showed high quality RNA Quality Numbers (RQN mean = 9.8). Library preparation was performed according to

the manufacturer's protocol using the Lexogen QuantSeq 3' mRNA-Seq Library Prep Kit FWD with UMI's (Lexogen, Austria). The input amount was 25 ng total RNA. Bead purified libraries were normalized and finally sequenced on the NextSeq2000 system (Illumina, USA) with a read setup of 1x100 bp. The BCL Convert Tool (version 3.8.4) was used to convert the bcl files to fastq files as well as for adapter trimming and demultiplexing.

Analysis of the mRNA sequencing data. Sequencing data was processed following the recommended protocol on the Lexogen website (https://faqs.lexogen.com/faq/quantseq-with-udi-v2). Using the umi_tools software package (version 1.1.4), we extracted the Unique molecular identifier (UMI) from the reads of the FASTQ files and removed the adjacent TATA spacer by the “umi_tools extract” command, resulting in FASTQ files with the UMI in the read names [153]. Since QuantSeq 3‘ mRNA-Seq reads contain part of the poly-A tail, we next trimmed any sequenced poly-A sequences and the Lexogen adapter sequences from the read ends, as well as low quality bases by cutadapt (version 3.5) [154]. Reads potentially containing rRNA transcripts were removed using the sortMeRNA tool (version 4.3.7), using the recommended latest database “smr_v4.3_default_db” [155]. Data quality was accessed at every step using the tools FASTQC and Multiqc.

For reads alignment, we generated a fused meta-genome with the STAR alignment tool (version 2.7.10a) [156], combining the genomic sequences and genome annotations of the mouse (GRCm39 ensembl version 111) and toxoplasma (ensemble protists version 55) reference from Ensembl. The reads were then aligned to the fused STAR genome index, that was generated setting the minimal splice site overlap to 99 (--sjdbOverhang 99). Uniquely mapped reads were selected from the BAM files using the samtools software package (version 1.13) [157]. Duplicated reads were then removed from the BAM files with umi_tools. Finally, gene count matrices were generated from the BAM files by the featurecount software (version 2.0.3) [158]. Differential gene expression analysis was performed on the gene count matrices by a custom R script, using the DESeq2 R package (version 1.44.0) [160]. The results of the differential gene expression analysis were then used for gene set enrichment analysis (GSEA), using the fgsea R package (version 1.30.0) [160].

Code and data availability. Raw sequencing data was submitted to the ENA database under accession number PRJNA1177674. Code that was used to analyze the data can be found on github at “https://github.com/caggtaagtat/QuantSeq-3-mRNA-sequencing”.

Statistical analysis. GraphPad Prism software (version 10) was used for data analysis. Symbols represent individual animals, columns represent mean values, and error bars represent the ± standard deviation (SD). Outliers were identified and excluded from the data using the ROUT test (Q = 1%) after which the Shapiro-Wilk test of normality was performed. Parametric data was analyzed using 2-way analysis of variance (ANOVA) corrected for multiple comparison using Tukey’s *post hoc* test.

Nonparametric data was analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Comparisons with a *P* value of ≥ 0.05 were considered statistically not significant and were not specifically marked.

Supplementary information

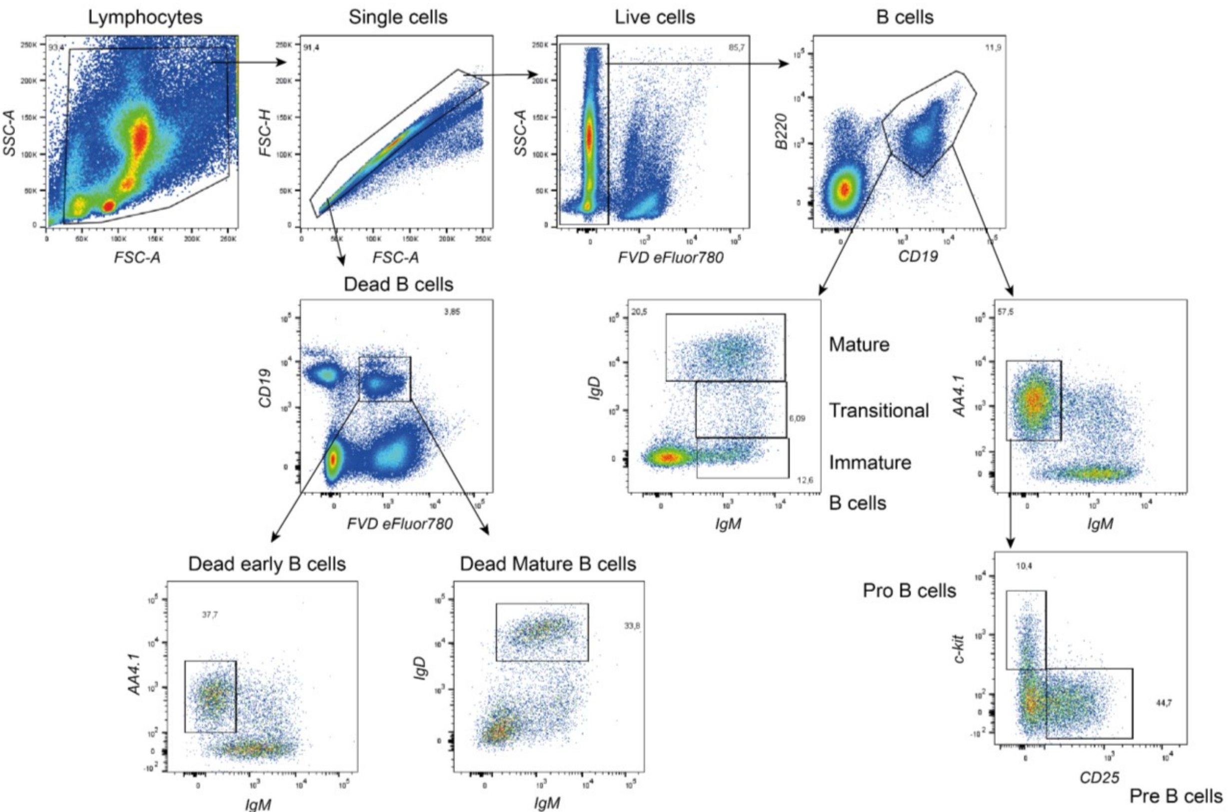


Figure S1: Gating strategy for the detection of B cell subpopulations in the BM.

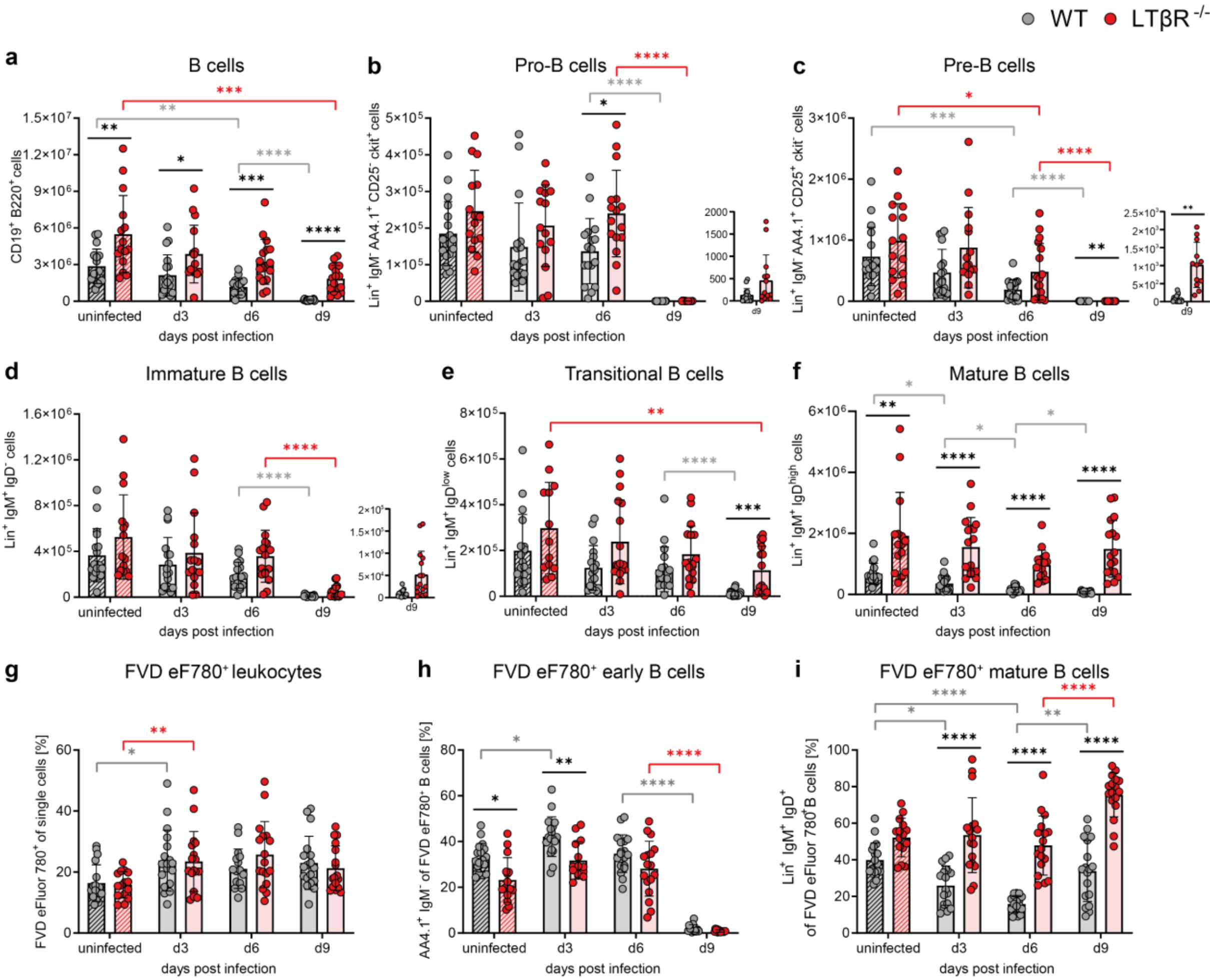


Figure S2: Absolute cell numbers and dead B cell subpopulation frequencies in the BM of LTβR$^{-/-}$ and WT mice during *T. gondii* infection. Using surface marker staining and flow cytometry, the following immune cell populations (gating strategy: Fig. S1) were identified in the BM of WT (n ≥ 12/group) and LTβR$^{-/-}$ (n ≥ 11/group) mice: **(a)** Pan-B cells (CD19$^+$ B220$^+$), **(b)** Pro-B cells (Lin$^+$ IgM$^-$ AA4.1$^+$ CD25$^-$ ckit$^+$), **(c)** Pre-B cells (Lin$^+$ IgM$^-$ AA4.1$^+$ CD25$^+$ ckit$^-$), **(d)** Immature B cells (Lin$^+$ IgM$^+$ IgD$^-$), **(e)** Transitional B cells (Lin$^+$ IgM$^-$ IgD^{low}), **(f)** Mature B cells (Lin$^+$ IgM$^-$ IgD^{high}), **(g)** dead leukocytes (FVD eFluor780$^+$, % of single cells), **(h)** dead early B cells (FVD eFluor780$^+$ CD19$^+$ AA4.1$^+$ IgM$^-$, % of dead B cells), and **(i)** dead mature B cells (FVD eFluor780$^+$ CD19$^+$ IgM$^+$ IgD$^+$, % of dead B cells). All data shown represent at least three independent experiments; symbols represent individual animals and columns represent mean values ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

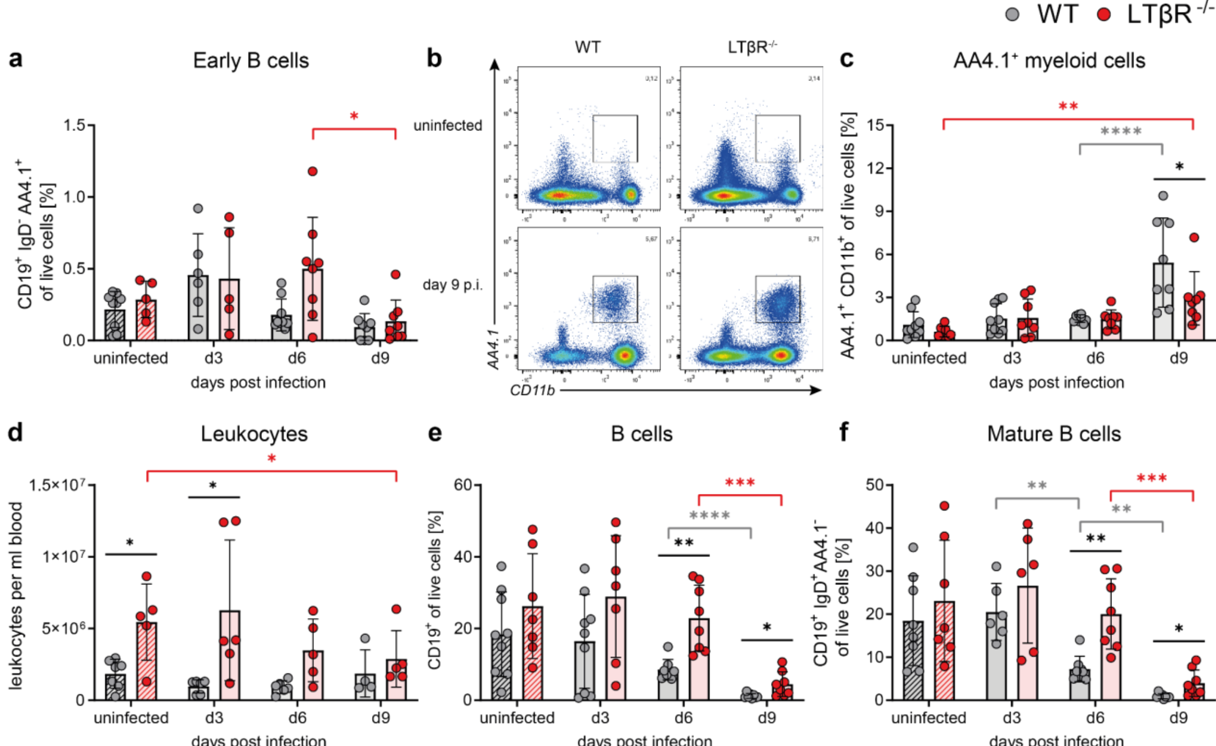


Figure S3: B cell populations and leukocyte counts in the blood of LTβR$^{-/-}$ and WT mice during *T. gondii* infection. Using surface marker staining and flow cytometry, the following immune cell populations were identified in the blood of WT (n ≥ 6/group) and LTβR$^{-/-}$ (n ≥ 6/group) mice and quantified as percentages of live cells: **(a)** early B cells (CD19^{+} AA4.1^{+} IgD^{-}) **(b & c)** AA4.1-positive myeloid cells (CD11b^{+}), (b) shows a set of representative images. **(d)** Counted blood leukocytes from WT (n ≥ 4/group) and LTβR$^{-/-}$ (n ≥ 5/group) mice. **(e)** Pan-B cells (CD19^{+}) **(f)** Mature B cells (CD19^{+} AA4.1^{-} IgD^{+}). All data shown represent at least two independent experiments; symbols represent individual animals and columns represent mean values ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

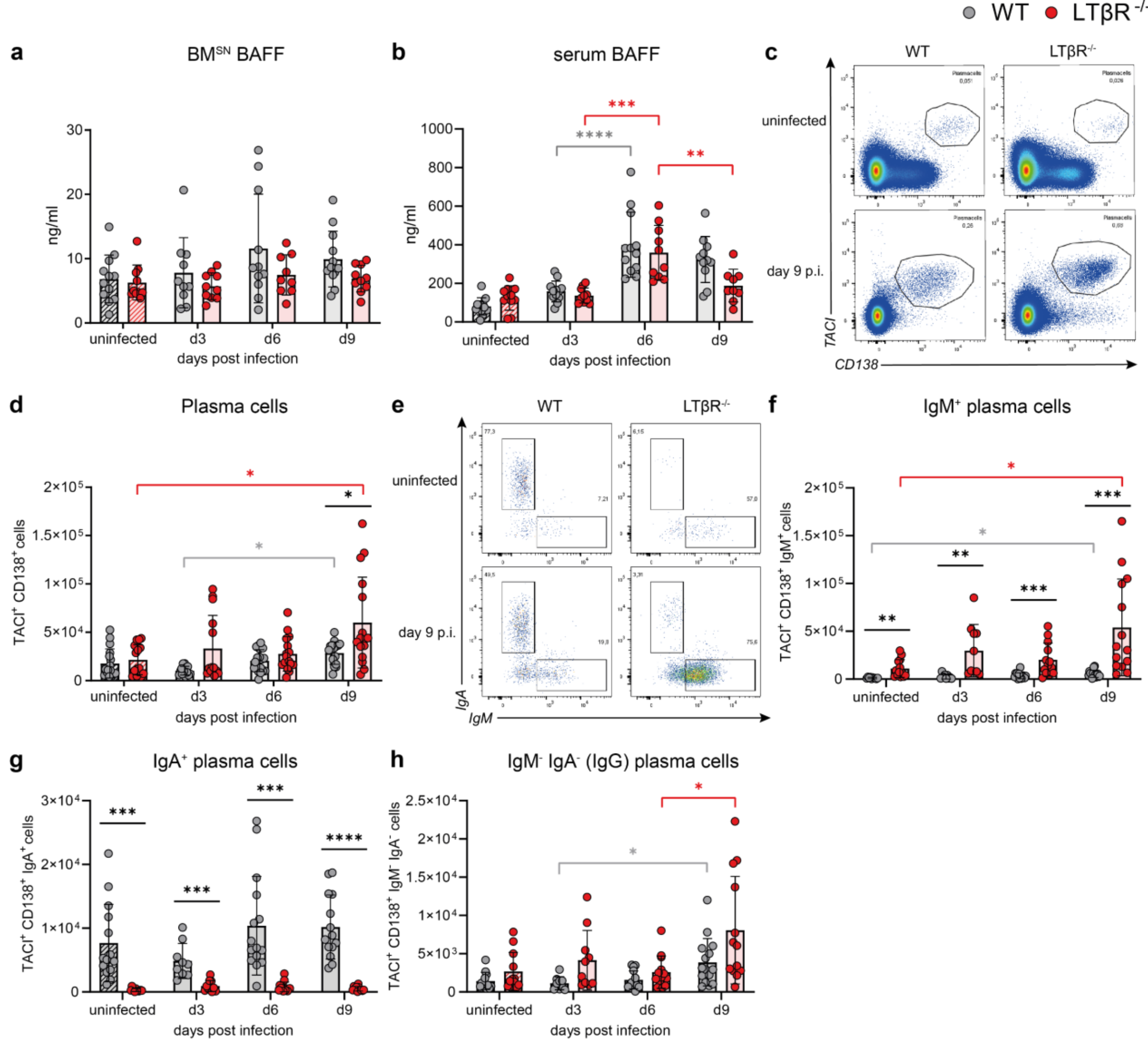


Figure S4: BAFF in BM^{SN} and serum, and BM plasma cells of LTβR$^{-/-}$ and WT mice during *T. gondii* infection. BAFF measured in the **(a)** BM^{SN} and **(b)** serum of uninfected and infected WT (n ≥ 10/group) and LTβR$^{-/-}$ (n ≥ 8/group) mice *via* a bead-based immunoassay (LegendPlex, BioLegend, USA). Using surface marker staining and flow cytometry, the following immune cell populations were identified in the BM of WT (n ≥ 9/group) and LTβR$^{-/-}$ (n ≥ 8/group) mice: **(c)** Representative images of plasma cell (TACI^{+} 138^{+}) gating, **(d)** absolute numbers of pan-plasma cells, **(e)** representative images of surface IgM and IgA plasma cell gating, and **(f – h)** absolute numbers of (f) IgM plasma cells, (g) IgA plasma cells and (h) IgM^{-} IgA^{-} (IgG) plasma cells in the BM. BM^{SN} = bone marrow supernatant. Data shown represent at least three independent experiments; symbols represent individual animals and columns represent mean values ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

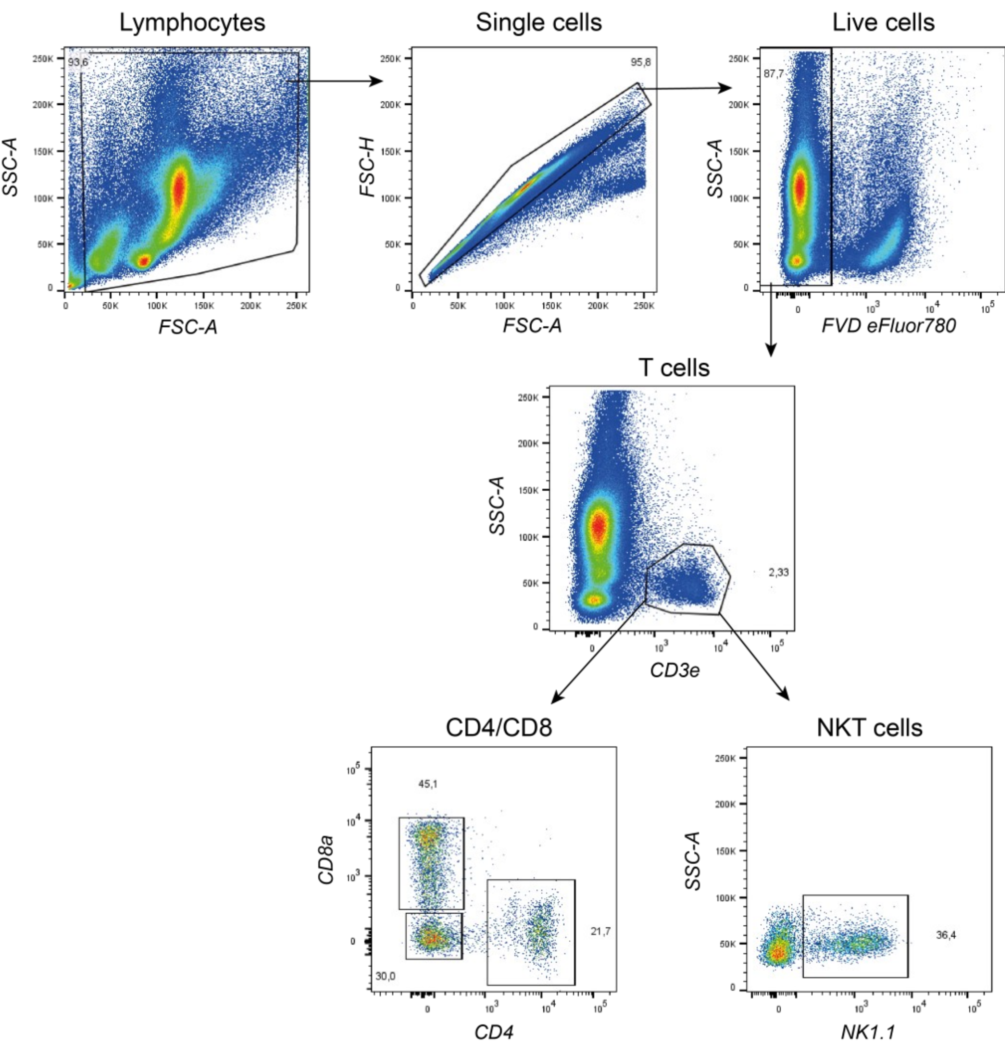


Figure S5: Gating strategy for the detection of T cell subpopulations in the BM.

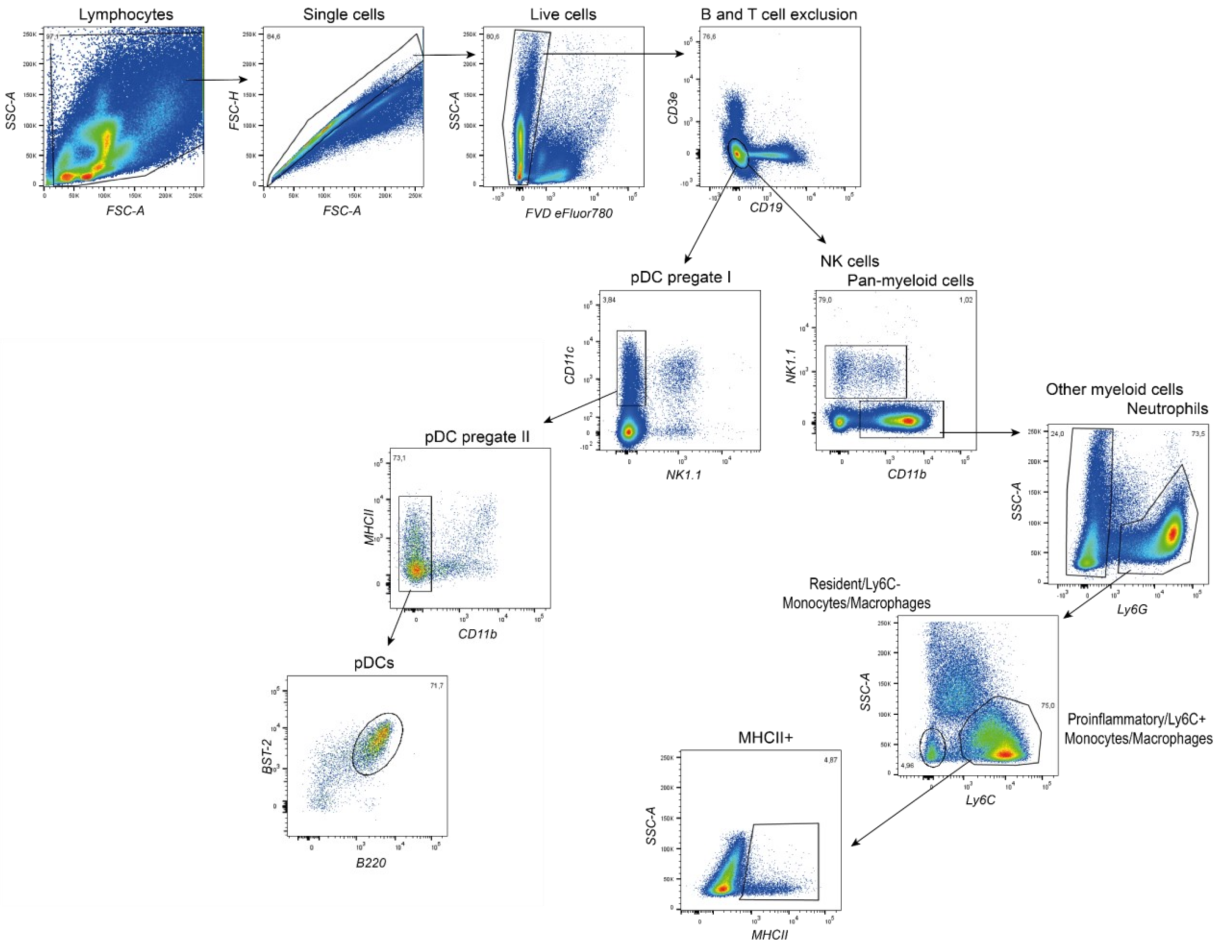


Figure S6: Gating strategy for the detection pDCs, NK cells, and myeloid subpopulations in the BM.

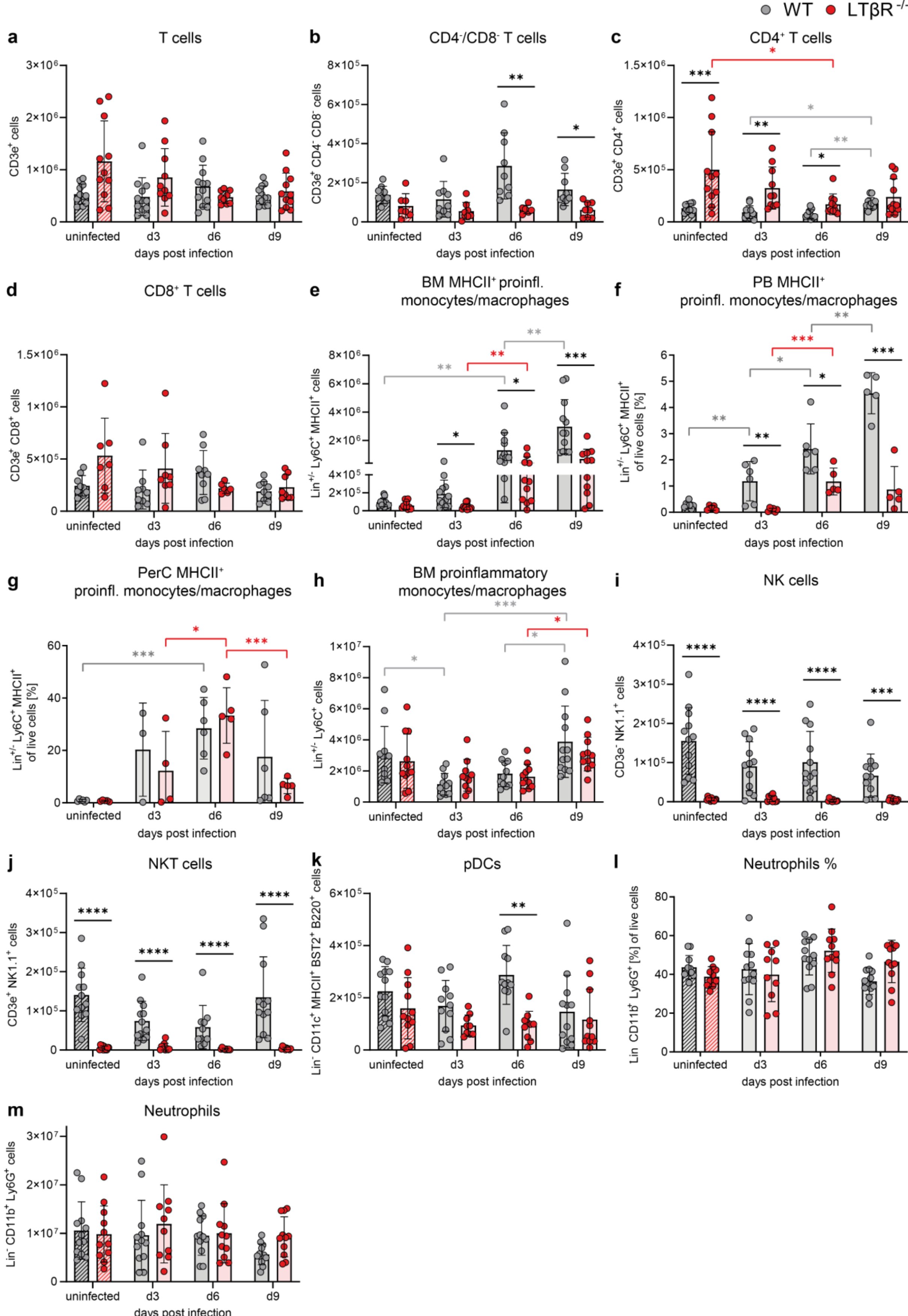


Figure S7: Absolute numbers of BM T cells, (MHCII⁺) proinflammatory monocytes/macrophages, NK cells, NKT cells, pDCs, and neutrophils in the BM, and MHCII⁺ proinflammatory monocytes/macrophages in the PB and PerC. Using surface marker staining and flow cytometry, the following immune cell populations (gating strategy:

Fig. S5, S6 and S8) were identified: in the BM of WT (n ≥ 9/group) and LTβR$^{-/-}$ (n ≥ 7/group) mice: **(a)** pan-T cells (CD3e$^+$), **(b)** double-negative T cells (CD3e$^+$ CD4$^-$ CD8$^-$), **(c)** CD4$^+$ T cells (CD3e$^+$ CD4$^+$), **(d)** CD8$^+$ T cells (CD3e$^+$ CD8$^+$), and **(e)** MHCII-positive proinflammatory monocytes/macrophages (CD19$^-$ CD3e$^-$ NK.1.$^-$ CD11b$^+$ Ly6G$^-$ Ly6C$^+$ MHCII$^+$). MHCII-positive proinflammatory monocytes/macrophages were also identified in the **(f)** PB and **(g)** PerC of WT and LTβR$^{-/-}$ mice (both n ≥ 5/group), reported as % of live cells. Absolute numbers of BM **(h)** proinflammatory monocytes/macrophages (CD19$^-$ CD3e$^-$ NK.1.$^-$ CD11b$^+$ Ly6G$^-$ Ly6C$^+$), **(i)** NK cells (CD19$^-$ CD3e$^-$ NK1.1$^+$), **(j)** NKT cells (CD3e$^+$ NK1.1$^+$) and **(k)** plasmacytoid DCs (pDCs; CD19$^-$ CD3e$^-$ NK.1.$^-$ CD11c$^+$ CD11b$^-$ MHCII$^+$ B220$^+$ BST-2$^+$). BM neutrophils (CD19$^-$ CD3e$^-$ NK.1.$^-$ CD11b$^+$ Ly6G$^+$) reported as **(l)** frequency of live cells and **(m)** absolute numbers. BM = bone marrow. PB = peripheral blood. PerC = peritoneal cavity. Data shown represent: four independent experiments (BM) and two independent experiments each for PB and for PerC; symbols represent individual animals and columns represent mean values ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

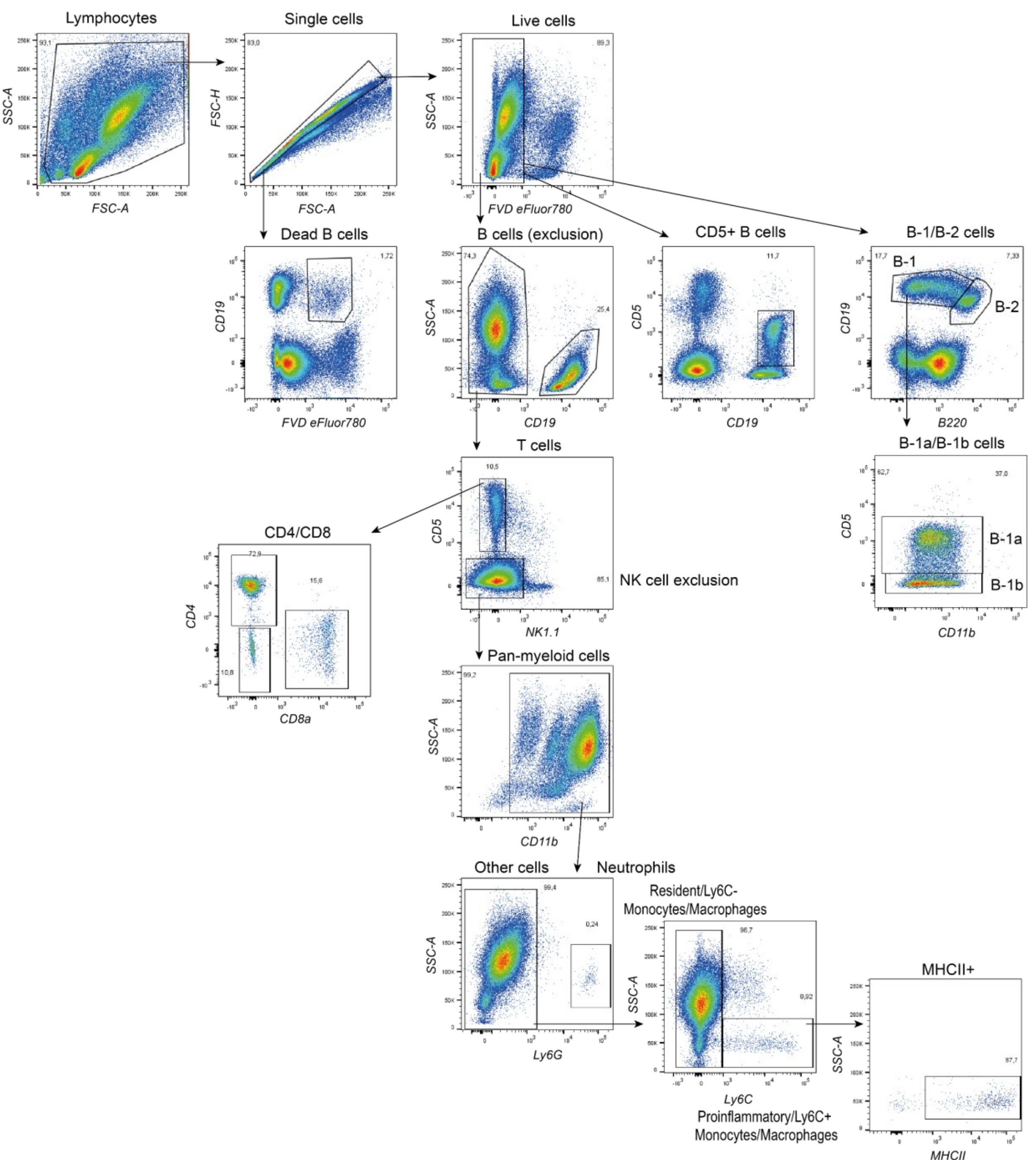
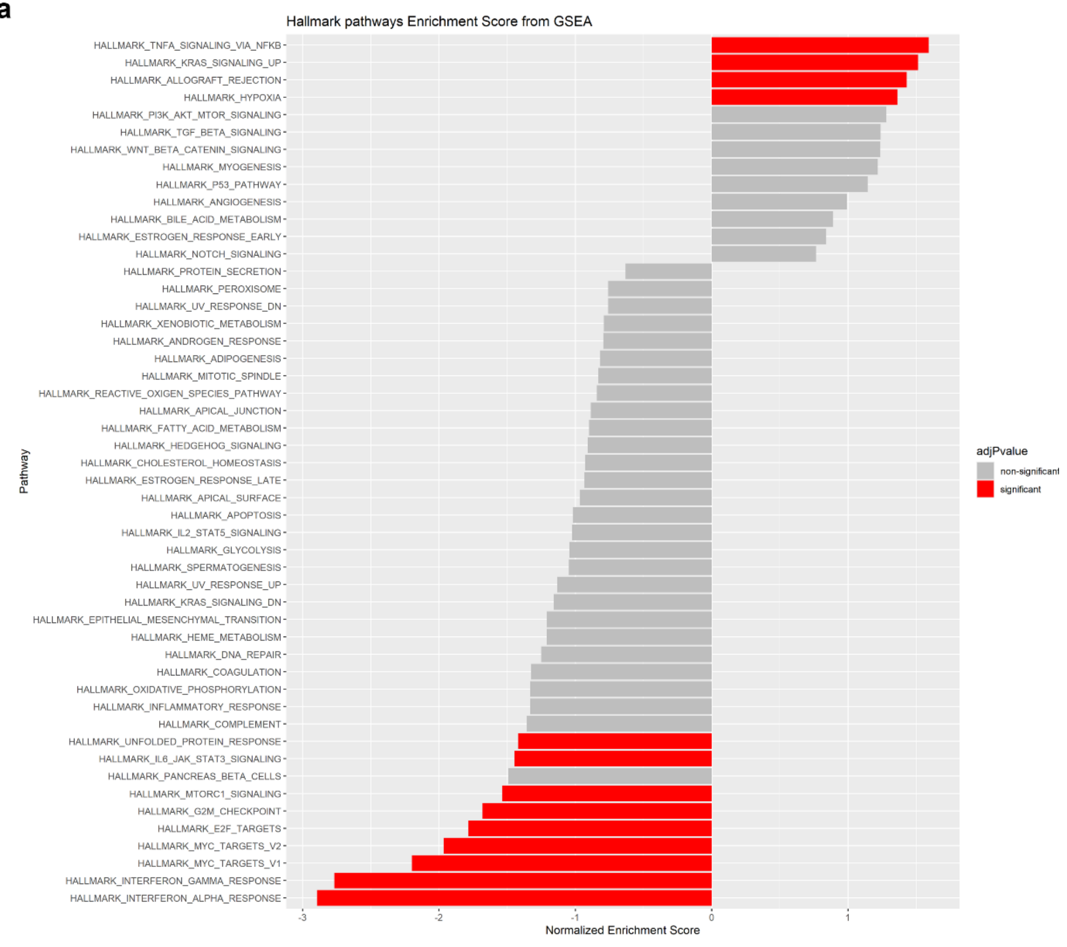
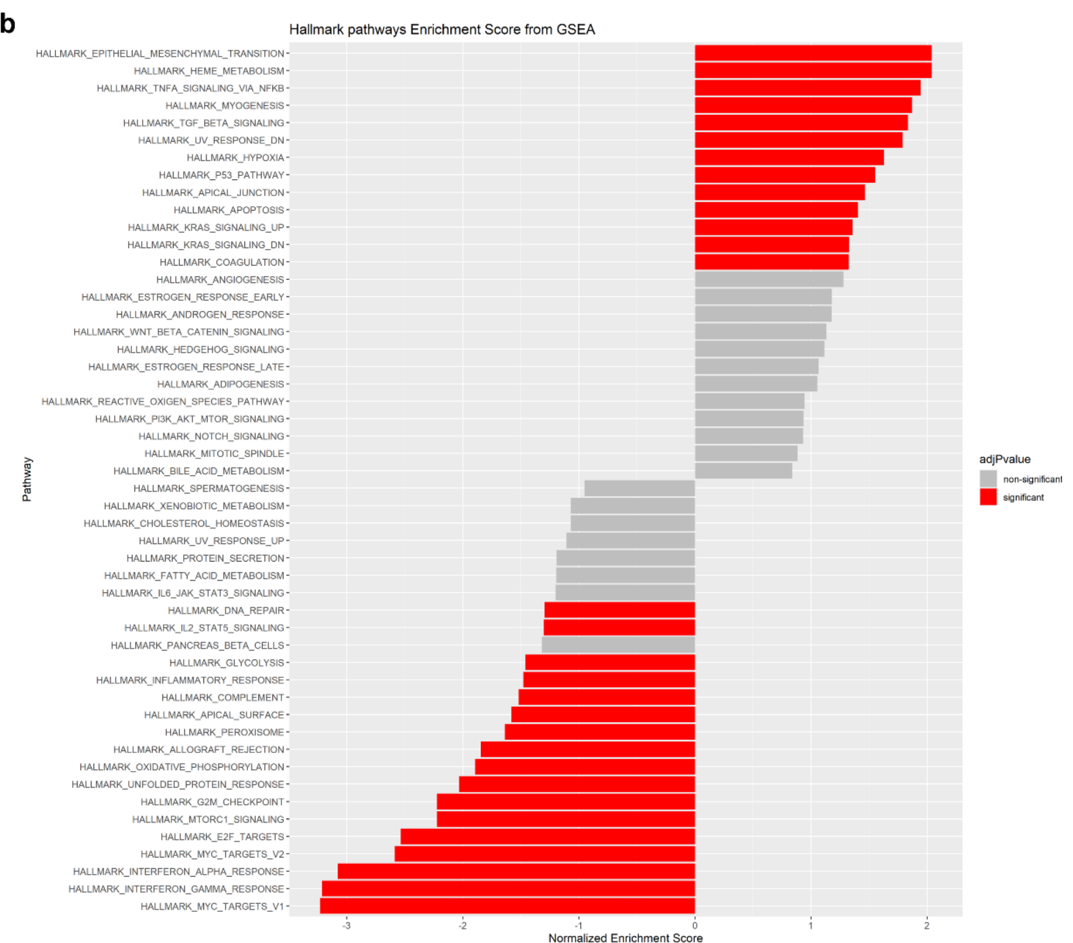


Figure S8: Gating strategy for the detection of B cells, T cells, and myeloid populations in the PerC.

a



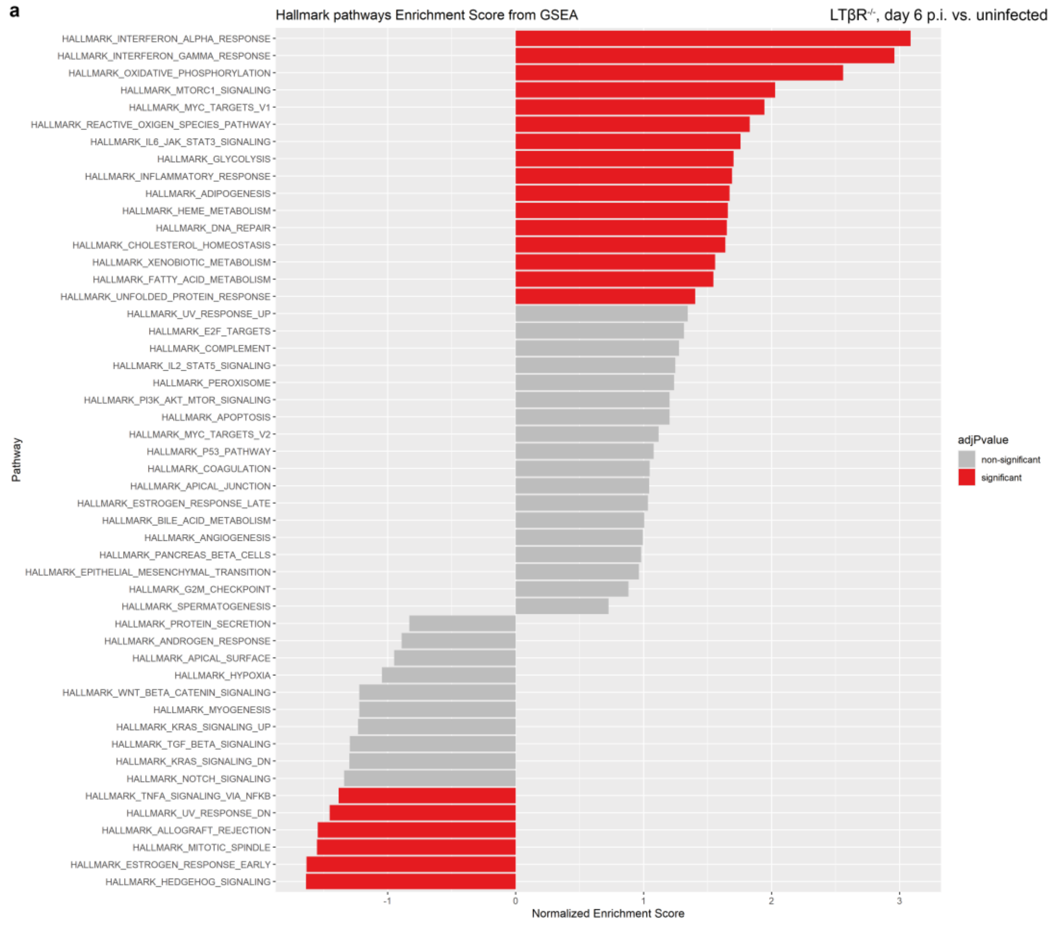
b

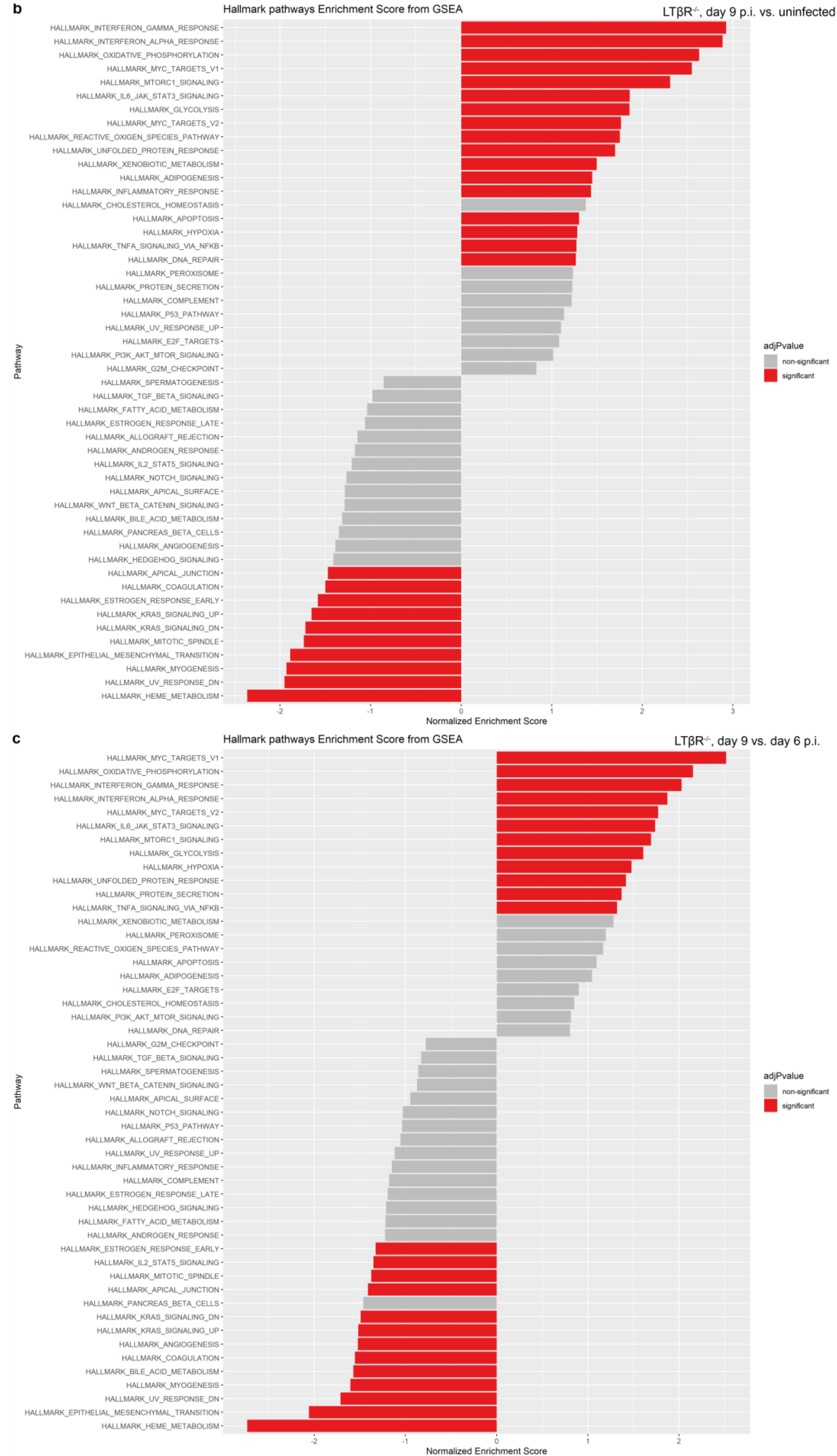


gene	name	counts					
		WT d9 I	WT d9 II	WT d9 III	LTβR-/- d9 I	LTβR-/- d9 II	LTβR-/- d9 III
TGME49_270250	GRA1	3	0	0	36	44	22
TGME49_286450	GRA5	0	0	1	34	27	17
TGME49_227620	GRA2	0	0	1	24	23	11
TGME49_261740	hypothetical protein 12	0	0	0	11	19	0
TGME49_203310	GRA7	0	0	0	6	13	11
TGME49_271050	SAG 34A/SAG/2	1	0	0	12	9	5
TGME49_227280	GRA3	0	0	0	11	14	2
TGME49_309590	ROP1	0	0	0	8	13	3
TGME49_233480	SRS29C	0	0	0	3	12	9
TGME49_213580	hypothetical protein	0	0	0	12	6	4

Figure S9: Complete GSEA genotype comparison and *T. gondii*-reads on day 9 p.i. GSEA of RNA sequencing data from BM samples of uninfected and *T. gondii*-infected WT and LTβR$^{-/-}$ mice (n = 3/group, except for LTβR$^{-/-}$ uninfected: n = 2). A positive NES value indicates gene set enrichment in the experimental condition, a negative NES value indicates gene set in the control condition, and a gray color describes non-significance (adjusted p-

value > 0.01). **(a)** Uninfected LTβR$^{-/-}$ vs WT controls, displaying enrichment/depletion in LTβR$^{-/-}$ BM. **(b)** LTβR$^{-/-}$ vs WT, day 6 p.i., displaying enrichment/depletion in LTβR$^{-/-}$ BM. **(c)** LTβR$^{-/-}$ vs WT, day 9 p.i., displaying enrichment/depletion in LTβR$^{-/-}$ BM. **(d)** NES of selected GSEA gene sets, summarized and represented as colors in a heat map. The left panel summarizes genotype comparisons (LTβR$^{-/-}$ vs. WT), displaying enrichment in the LTβR$^{-/-}$ compared to WT controls. The middle panel summarizes intra-WT comparisons: d6 and 9 p.i. vs. uninfected controls, displaying enrichment in infected animals; and d9 vs. d6, displaying enrichment on day 9 p.i. compared to the day 6 p.i. control. Similarly, the right panel summarizes intra-LTβR$^{-/-}$ comparisons: d6 and 9 p.i. vs. uninfected controls, displaying enrichment in infected animals; and d9 vs. d6, displaying enrichment on day 9 p.i. compared to the day 6 p.i. control. **(e)** Absolute counts of *T. gondii*-derived transcripts found in the BM of infected WT and LTβR$^{-/-}$ mice on day 9 p.i.




b
Hallmark pathways Enrichment Score from GSEA
LTβR-/-, day 9 p.i. vs. uninfected
HALLMARK_INTERFERON_GAMMA_RESPONSE
HALLMARK_INTERFERON_ALPHA_RESPONSE
HALLMARK_OXIDATIVE_PHOSPHORYLATION
HALLMARK_MYC_TARGETS_V1
HALLMARK_MTORC1_SIGNALING
HALLMARK_IL6_JAK_STAT3_SIGNALING
HALLMARK_GLYCOLYSIS
HALLMARK_MYC_TARGETS_V2
HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY
HALLMARK_UNFOLDED_PROTEIN_RESPONSE
HALLMARK_XENOBIOTIC_METABOLISM
HALLMARK_ADIPOGENESIS
HALLMARK_INFLAMMATORY_RESPONSE
HALLMARK_CHOLESTEROL_HOMEOSTASIS
HALLMARK_APOPTOSIS
HALLMARK_HYPOXIA
HALLMARK_TNFA_SIGNALING_VIA_NFKB
HALLMARK_DNA_REPAIR
HALLMARK_PEROXISOME
HALLMARK_PROTEIN_SECRETION
HALLMARK_COMPLEMENT
HALLMARK_P53_PATHWAY
HALLMARK_UV_RESPONSE_UP
HALLMARK_E2F_TARGETS
HALLMARK_PI3K_AKT_MTOR_SIGNALING
HALLMARK_G2M_CHECKPOINT
HALLMARK_SPERMATOGENESIS
HALLMARK_TGF_BETA_SIGNALING
HALLMARK_FATTY_ACID_METABOLISM
HALLMARK_ESTROGEN_RESPONSE_LATE
HALLMARK_ALLOGRAFT_REJECTION
HALLMARK_ANDROGEN_RESPONSE
HALLMARK_IL2_STAT5_SIGNALING
HALLMARK_NOTCH_SIGNALING
HALLMARK_APICAL_SURFACE
HALLMARK_WNT_BETA_CATENIN_SIGNALING
HALLMARK_BILE_ACID_METABOLISM
HALLMARK_PANCREAS_BETA_CELLS
HALLMARK_ANGIOGENESIS
HALLMARK_HEDGEHOG_SIGNALING
HALLMARK_APICAL_JUNCTION
HALLMARK_COAGULATION
HALLMARK_ESTROGEN_RESPONSE_EARLY
HALLMARK_KRAS_SIGNALING_UP
HALLMARK_KRAS_SIGNALING_DN
HALLMARK_MITOTIC_SPINDLE
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
HALLMARK_MYOGENESIS
HALLMARK_UV_RESPONSE_DN
HALLMARK_HEME_METABOLISM
Pathway
-2
-1
0
1
2
3
Normalized Enrichment Score
adjPvalue
non-significant
significant
c
Hallmark pathways Enrichment Score from GSEA
LTβR-/-, day 9 vs. day 6 p.i.
HALLMARK_MYC_TARGETS_V1
HALLMARK_OXIDATIVE_PHOSPHORYLATION
HALLMARK_INTERFERON_GAMMA_RESPONSE
HALLMARK_INTERFERON_ALPHA_RESPONSE
HALLMARK_MYC_TARGETS_V2
HALLMARK_IL6_JAK_STAT3_SIGNALING
HALLMARK_MTORC1_SIGNALING
HALLMARK_GLYCOLYSIS
HALLMARK_HYPOXIA
HALLMARK_UNFOLDED_PROTEIN_RESPONSE
HALLMARK_PROTEIN_SECRETION
HALLMARK_TNFA_SIGNALING_VIA_NFKB
HALLMARK_XENOBIOTIC_METABOLISM
HALLMARK_PEROXISOME
HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY
HALLMARK_APOPTOSIS
HALLMARK_ADIPOGENESIS
HALLMARK_E2F_TARGETS
HALLMARK_CHOLESTEROL_HOMEOSTASIS
HALLMARK_PI3K_AKT_MTOR_SIGNALING
HALLMARK_DNA_REPAIR
HALLMARK_G2M_CHECKPOINT
HALLMARK_TGF_BETA_SIGNALING
HALLMARK_SPERMATOGENESIS
HALLMARK_WNT_BETA_CATENIN_SIGNALING
HALLMARK_APICAL_SURFACE
HALLMARK_NOTCH_SIGNALING
HALLMARK_P53_PATHWAY
HALLMARK_ALLOGRAFT_REJECTION
HALLMARK_UV_RESPONSE_UP
HALLMARK_INFLAMMATORY_RESPONSE
HALLMARK_COMPLEMENT
HALLMARK_ESTROGEN_RESPONSE_LATE
HALLMARK_HEDGEHOG_SIGNALING
HALLMARK_FATTY_ACID_METABOLISM
HALLMARK_ANDROGEN_RESPONSE
HALLMARK_ESTROGEN_RESPONSE_EARLY
HALLMARK_IL2_STAT5_SIGNALING
HALLMARK_MITOTIC_SPINDLE
HALLMARK_APICAL_JUNCTION
HALLMARK_PANCREAS_BETA_CELLS
HALLMARK_KRAS_SIGNALING_DN
HALLMARK_KRAS_SIGNALING_UP
HALLMARK_ANGIOGENESIS
HALLMARK_COAGULATION
HALLMARK_BILE_ACID_METABOLISM
HALLMARK_MYOGENESIS
HALLMARK_UV_RESPONSE_DN
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
HALLMARK_HEME_METABOLISM
Pathway
-2
-1
0
1
2
Normalized Enrichment Score
adjPvalue
non-significant
significant

d
Hallmark pathways Enrichment Score from GSEA
WT, day 6 p.i. vs. uninfected
HALLMARK_TNFA_SIGNALING_VIA_NFKB
HALLMARK_KRAS_SIGNALING_UP
HALLMARK_ALLOGRAFT_REJECTION
HALLMARK_HYPOXIA
HALLMARK_PI3K_AKT_MTOR_SIGNALING
HALLMARK_TGF_BETA_SIGNALING
HALLMARK_WNT_BETA_CATENIN_SIGNALING
HALLMARK_MYOGENESIS
HALLMARK_P53_PATHWAY
HALLMARK_ANGIOGENESIS
HALLMARK_BILE_ACID_METABOLISM
HALLMARK_ESTROGEN_RESPONSE_EARLY
HALLMARK_NOTCH_SIGNALING
HALLMARK_PROTEIN_SECRETION
HALLMARK_PEROXISOME
HALLMARK_UV_RESPONSE_DN
HALLMARK_XENOBIOTIC_METABOLISM
HALLMARK_ANDROGEN_RESPONSE
HALLMARK_ADIPOGENESIS
HALLMARK_MITOTIC_SPINDLE
HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY
HALLMARK_APICAL_JUNCTION
HALLMARK_FATTY_ACID_METABOLISM
HALLMARK_HEDGEHOG_SIGNALING
HALLMARK_CHOLESTEROL_HOMEOSTASIS
HALLMARK_ESTROGEN_RESPONSE_LATE
HALLMARK_APICAL_SURFACE
HALLMARK_APOPTOSIS
HALLMARK_IL2_STAT5_SIGNALING
HALLMARK_GLYCOLYSIS
HALLMARK_SPERMATOGENESIS
HALLMARK_UV_RESPONSE_UP
HALLMARK_KRAS_SIGNALING_DN
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
HALLMARK_HEME_METABOLISM
HALLMARK_DNA_REPAIR
HALLMARK_COAGULATION
HALLMARK_OXIDATIVE_PHOSPHORYLATION
HALLMARK_INFLAMMATORY_RESPONSE
HALLMARK_COMPLEMENT
HALLMARK_UNFOLDED_PROTEIN_RESPONSE
HALLMARK_IL6_JAK_STAT3_SIGNALING
HALLMARK_PANCREAS_BETA_CELLS
HALLMARK_MTORC1_SIGNALING
HALLMARK_G2M_CHECKPOINT
HALLMARK_E2F_TARGETS
HALLMARK_MYC_TARGETS_V2
HALLMARK_MYC_TARGETS_V1
HALLMARK_INTERFERON_GAMMA_RESPONSE
HALLMARK_INTERFERON_ALPHA_RESPONSE
Pathway
-3
-2
-1
0
1
Normalized Enrichment Score
adjPvalue
non-significant
significant
e
Hallmark pathways Enrichment Score from GSEA
WT, day 9 p.i. vs. uninfected
HALLMARK_OXIDATIVE_PHOSPHORYLATION
HALLMARK_INTERFERON_GAMMA_RESPONSE
HALLMARK_INTERFERON_ALPHA_RESPONSE
HALLMARK_GLYCOLYSIS
HALLMARK_MTORC1_SIGNALING
HALLMARK_MYC_TARGETS_V1
HALLMARK_UNFOLDED_PROTEIN_RESPONSE
HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY
HALLMARK_PROTEIN_SECRETION
HALLMARK_IL6_JAK_STAT3_SIGNALING
HALLMARK_PEROXISOME
HALLMARK_XENOBIOTIC_METABOLISM
HALLMARK_COMPLEMENT
HALLMARK_ALLOGRAFT_REJECTION
HALLMARK_MYC_TARGETS_V2
HALLMARK_ADIPOGENESIS
HALLMARK_PI3K_AKT_MTOR_SIGNALING
HALLMARK_INFLAMMATORY_RESPONSE
HALLMARK_FATTY_ACID_METABOLISM
HALLMARK_UV_RESPONSE_UP
HALLMARK_APOPTOSIS
HALLMARK_P53_PATHWAY
HALLMARK_HYPOXIA
HALLMARK_CHOLESTEROL_HOMEOSTASIS
HALLMARK_DNA_REPAIR
HALLMARK_E2F_TARGETS
HALLMARK_APICAL_SURFACE
HALLMARK_ANGIOGENESIS
HALLMARK_BILE_ACID_METABOLISM
HALLMARK_G2M_CHECKPOINT
HALLMARK_IL2_STAT5_SIGNALING
HALLMARK_ESTROGEN_RESPONSE_LATE
HALLMARK_TGF_BETA_SIGNALING
HALLMARK_TNFA_SIGNALING_VIA_NFKB
HALLMARK_WNT_BETA_CATENIN_SIGNALING
HALLMARK_COAGULATION
HALLMARK_ANDROGEN_RESPONSE
HALLMARK_APICAL_JUNCTION
HALLMARK_KRAS_SIGNALING_UP
HALLMARK_PANCREAS_BETA_CELLS
HALLMARK_ESTROGEN_RESPONSE_EARLY
HALLMARK_SPERMATOGENESIS
HALLMARK_MYOGENESIS
HALLMARK_MITOTIC_SPINDLE
HALLMARK_NOTCH_SIGNALING
HALLMARK_HEDGEHOG_SIGNALING
HALLMARK_KRAS_SIGNALING_DN
HALLMARK_UV_RESPONSE_DN
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
HALLMARK_HEME_METABOLISM
Pathway
-2
-1
0
1
2
Normalized Enrichment Score
adjPvalue
non-significant
significant

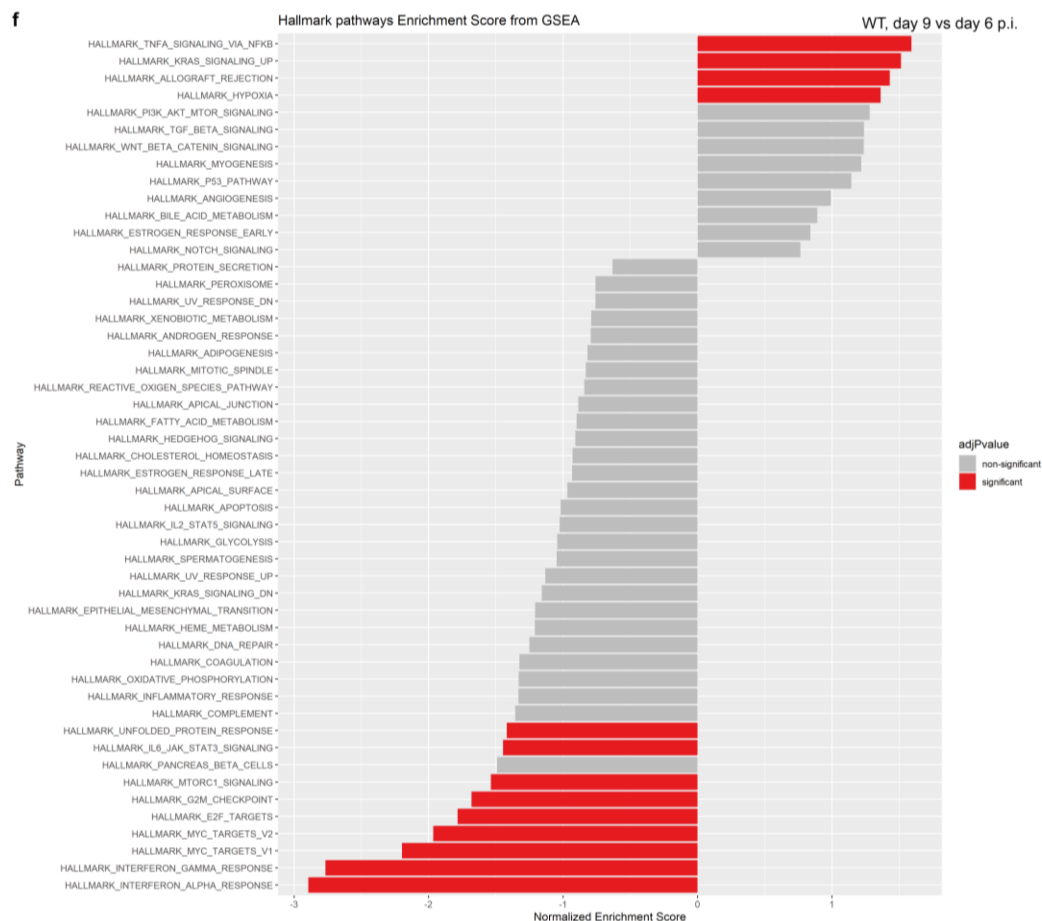


Figure S10: Complete GSEA of intra-genotype comparisons during *T. gondii* infection. GSEA of RNA sequencing data from BM samples of uninfected and *T. gondii*-infected WT and LTβR$^{-/-}$ mice (n = 3/group, except for LTβR$^{-/-}$ uninfected: n = 2). A positive NES value indicates gene set enrichment in the experimental condition, a negative NES value indicates gene set enrichment in the control condition, and a gray color describes non-significance (adjusted p-value > 0.01). **(a)** LTβR$^{-/-}$ day 6 p.i. and **(b)** LTβR$^{-/-}$ day 9 p.i. vs uninfected controls, displaying enrichment/depletion in infected LTβR$^{-/-}$ BM. **(c)** LTβR$^{-/-}$ day 9 vs day 6 p.i., displaying enrichment/depletion on day 9 p.i. **(d)** WT day 6 p.i. and **(e)** WT day 9 p.i. vs uninfected controls, displaying enrichment/depletion in infected WT BM. **(f)** WT day 9 vs day 6 p.i., displaying enrichment/depletion on day 9 p.i.

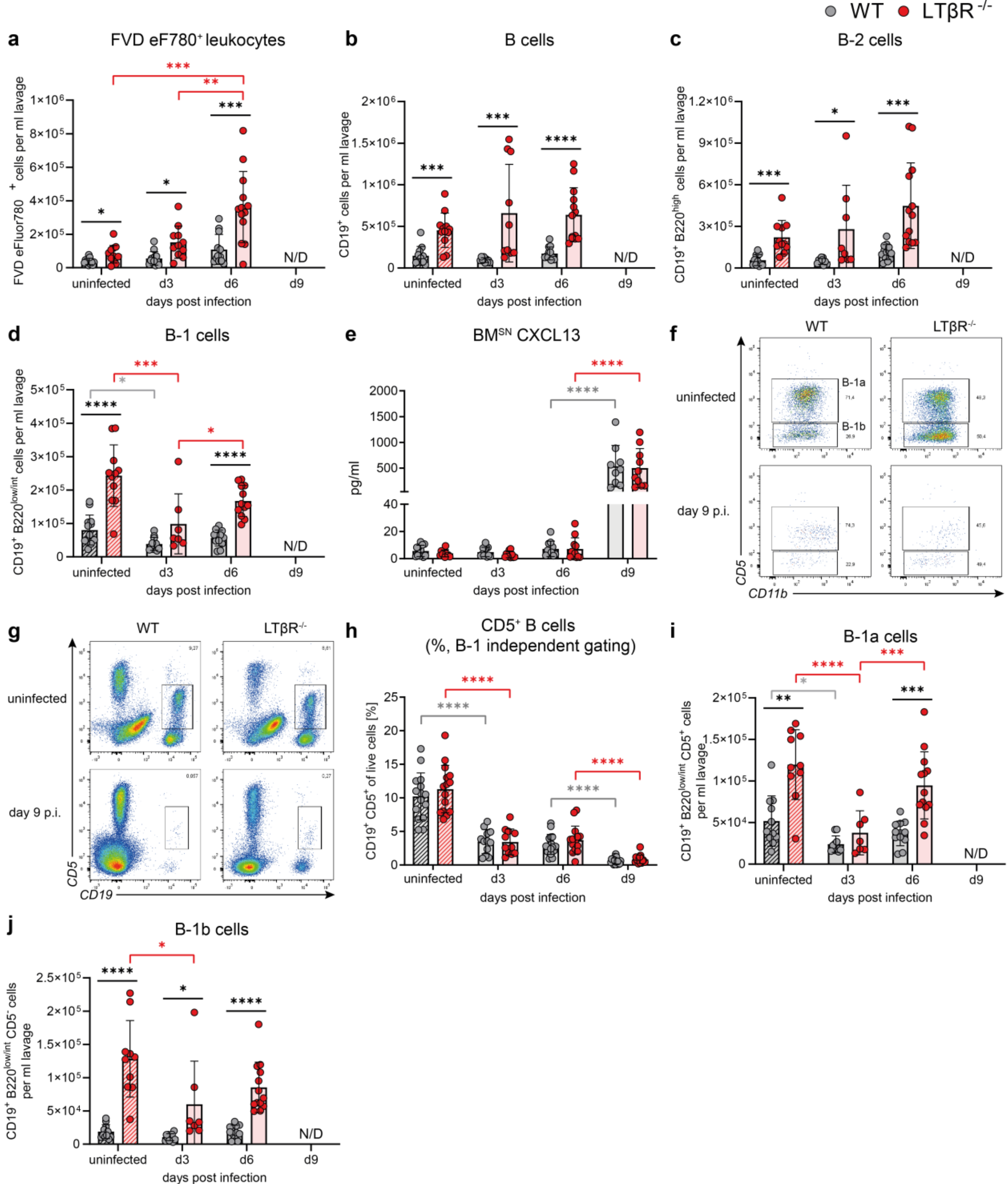


Figure S11: Absolute numbers of B cell subpopulations and direct B-1a gating in the PerC of *T. gondii*-infected WT and LTβR$^{-/-}$ mice. Using surface marker staining and flow cytometry, the following immune cell populations (gating strategy: Fig. S8) were identified in the PerC of WT (n ≥ 12/group) and LTβR$^{-/-}$ (n ≥ 10/group) mice: absolute numbers of **(a)** dead leukocytes (FVD eFluor780^{+}), **(b)** B cells (CD19^{+}), **(c)** B-2 cells (CD19^{+} B220^{high}), and **(d)** B-1 cells (CD19^{+} B220$^{low/int}$) per ml peritoneal lavage. **(e)** CXCL13 measured in the BM^{SN} of uninfected and infected WT (n ≥ 9/group) and LTβR$^{-/-}$ (n ≥ 8/group) mice *via* a bead-based immunoassay (LegendPlex, BioLegend, USA). **(f)** B-1a (CD5^{+}) and B-1b (CD5^{-}) cell gating in a set of representative images. **(g & h)** Alternative, direct detection of B-1a cells (CD19^{+} CD5^{+}) independent of prior B-1 (CD19^{+} B220$^{low/int}$) cell gating. (g) shows a set of representative images. **(i)** Absolute numbers of B-1a cells (CD19^{+} B220$^{low/int}$ CD5^{+}) and **(j)** B-1b cells (CD19^{+} B220$^{low/int}$ CD5^{-}) per ml peritoneal lavage. No reliable cell counts could be determined for day 9 p.i. due to extensive cell debris and clumping in the peritoneal lavages. While excluding non-single-cell events during flow cytometry allowed for the determination of live cell frequencies, absolute immune cell numbers could not be assessed on day 9 p.i.. PerC = peritoneal cavity. BM^{SN} = bone marrow supernatant. All data shown represent at least three independent

experiments; symbols represent individual animals and columns represent mean values ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

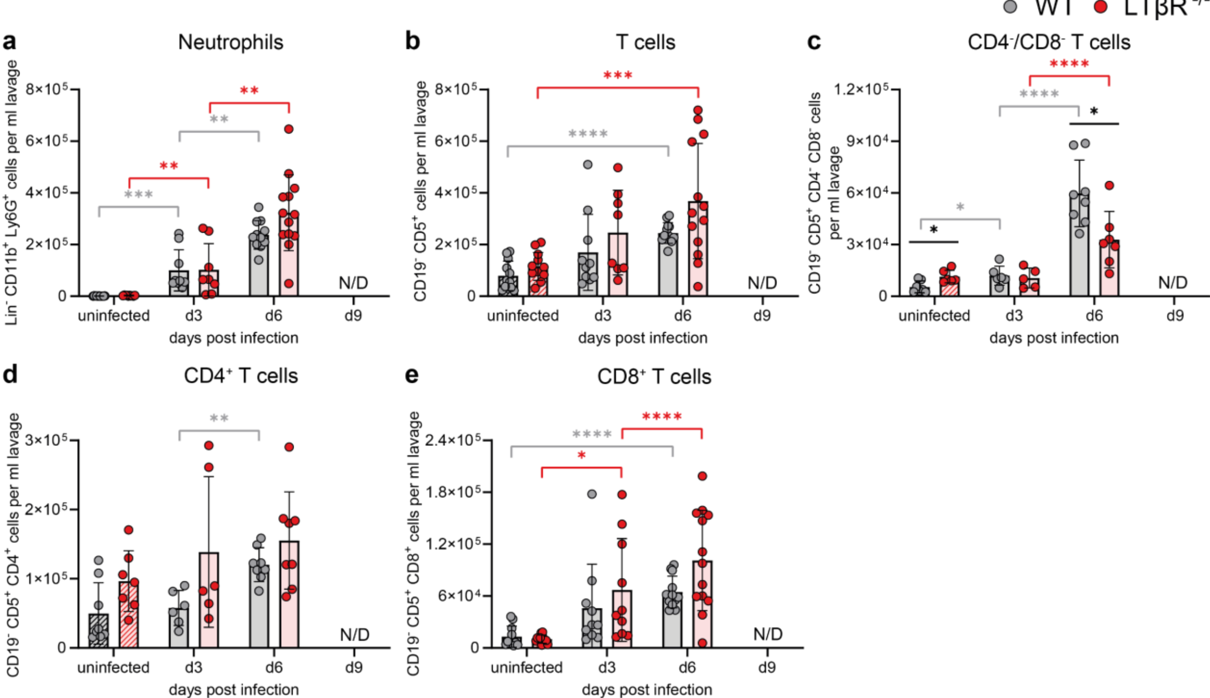


Figure S12: Absolute numbers of neutrophils and T cell subsets in the PerC of *T. gondii*-infected WT and LTβR$^{-/-}$ mice. Using surface marker staining and flow cytometry, the following immune cell populations (gating strategy: Fig. S8) were identified in the PerC of WT (n ≥ 12/group) and LTβR$^{-/-}$ (n ≥ 10/group) mice: absolute numbers of **(a)** neutrophils (CD19$^-$ CD5$^-$ NK1.1$^-$ CD11b$^+$ Ly6G$^+$), **(b)** T cells (CD19$^-$ CD5$^+$), **(c)** double negative T cells (CD19$^-$ CD5$^+$ CD4$^-$ CD8$^-$), **(d)** CD4$^+$ T cells (CD19$^-$ CD5$^+$ CD4$^+$), and **(e)** CD8$^+$ T cells (CD19$^-$ CD5$^+$ CD8$^+$). For (c) and (d): WT: n ≥ 6/group) and LTβR$^{-/-}$: n ≥ 5/group. No reliable cell counts could be determined for day 9 p.i. due to extensive cell debris and clumping in the peritoneal lavages. While excluding non-single-cell events during flow cytometry allowed for the determination of live cell frequencies, absolute immune cell numbers could not be assessed on day 9 p.i.. PerC = peritoneal cavity. All data shown represent at least two independent experiments; symbols represent individual animals and columns represent mean values ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

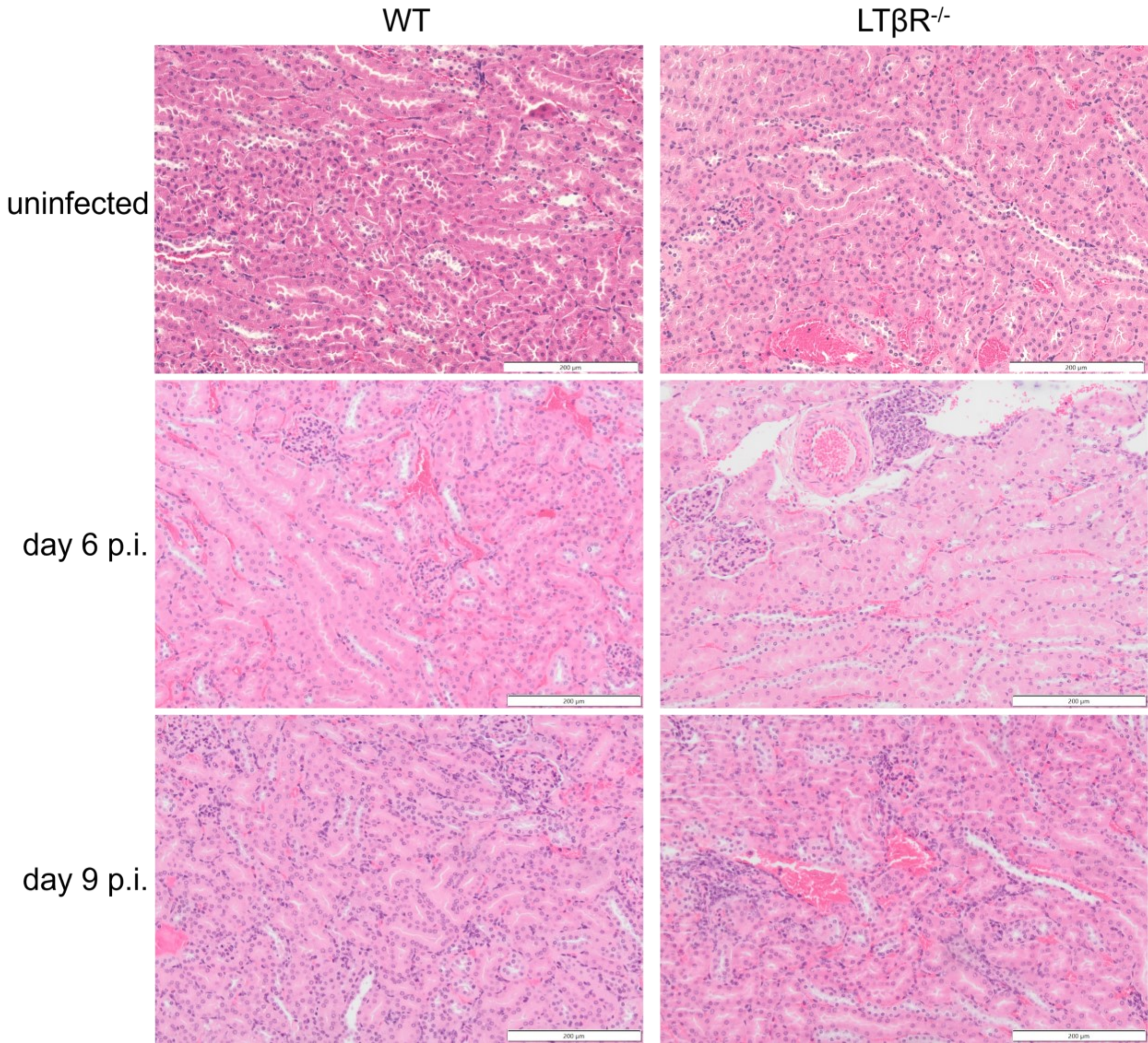


Figure S13: Histological analysis of kidneys in WT and LTβR$^{-/-}$ mice during *T. gondii* infection. Representative H&E images of kidneys from WT and LTβR$^{-/-}$ mice before and during *T. gondii* infection. n = 3 per genotype, except for LTβR$^{-/-}$ uninfected mice (n = 1). Scale bars: 200 µm.

Antibodies	Clone	Fluorochromes	Final dilution*	Manufacturer
AA4.1 (CD93)	AA4.1	PE	1:150	BioLegend
BST-2 (CD317, PDCA1)	927	BV650	1:150	BioLegend
B220 (CD45R)	RA3-6B2	PerCP-Cy5.5	1:300	BioLegend
B220 (CD45R)	RA3-6B2	FITC	1:150	BD Biosciences
CD3e	145-2C11	BV711	1:150	BioLegend
CD3e	145-2C11	FITC	1:150	BD Biosciences
CD4	GK1.5	PerCP-Cy5.5	1:200	BioLegend
CD5	53-7.3	PE	1:400	BioLegend
CD8a	53/6.7	BV711	1:150	BioLegend
CD8a	53/6.7	APC	1:150	BD Biosciences
CD11b	M1/70	BV510, AlexaFluor 700	1:200	BioLegend
CD11c	HL3	PE-Cy7	1:200	BD Biosciences
CD16/32	93	-	1:100	eBioscience
CD19	6D5	BV421, BV510, BV605, BV785	1:150 (BV510, BV785) 1:200 (BV421, BV605)	BioLegend

CD25	PC61	PE-Cy7	1:800	BioLegend
c-kit (CD117)	2B8	BV650	1:600	BioLegend
IgA	- (polyclonal)	AlexaFluor 647	1:10,000	SouthernBiotech
IgD	11-26c.2a	AlexaFluor 647	1:1400	BioLegend
IgM	RMM-1	FITC	1:150	BioLegend
Ly6C	HK1.4	BV605	1:200	BioLegend
Ly6G	1A8	PerCP-Cy5.5, BV421	1:200	BioLegend
MHCII (I-A/I-E)	M5/114.15.2	BV510	1:200	BioLegend
NK1.1	PK136	BV421, BV650	1:200	BioLegend
CD138 (Syndecan-1)	281-2	PE	1:1500	BioLegend
TACI (CD267)	8F10	BV421	1:500	BD Biosciences
-	-	Fixable Viability Dye (FVD) eFluor780	1:5000	eBioscience

Supplementary Table 1: Antibody-conjugates and dyes utilized in the flow cytometry analysis. Final dilution refers to antibody dilution after addition to cells incubated in Fc-block (CD16/32) solution. APC = Allophycocyanin, BV = Brilliant Violet, Cy = Cyanine, FITC = Fluorescein, PE = Phycoerythrin, PerCP = Peridinin-Chlorophyll-protein.

	Name	**Marker**	**Gating strategy supplementary figure**
General	live cells	FVD eFluor780-	All
	dead cells	FVD eFluor780+	All
Bone marrow	**B cells**	CD19+ B220+	1
	pro-B cells	CD19+ B220+ AA4.1+ IgM- c-kit+ CD25-	1
	pre-Bcells	CD19+ B220+ AA4.1+ IgM- c-kit- CD25+	1
	immature B cells	CD19+ B220+ IgM+ IgD-	1
	transitional B cells	CD19+ B220+ IgM+ IgD(low)	1
	mature B cells	CD19+ B220+ IgM+ IgD(high)	1
	dead B cells	FVD eFluor780+ CD19+	1
	dead early B cells	FVD eFluor780+ CD19+ AA4.1+ IgM-	1
	dead mature B cells	FVD eFluor780+ CD19+ IgM+ IgD+	1
	plasma cells	TACI+ CD138+	-
	IgM+ plasma cells	TACI+ CD138+ IgM+ IgA-	-
	IgA+ plasma cells	TACI+ CD138+ IgM- IgA+	-
	IgM- IgA- (IgG) plasma cells	TACI+ CD138+ IgM- IgA-	-
	T cells	CD3e+	5
	CD4+ T cells	CD3e+ CD4+ CD8a-	5
	CD8+ T cells	CD3e+ CD4- CD8a+	5
	double-negative T cells	CD3e+ CD4- CD8a-	5
	NKT cells	CD3e+ NK1.1+	5
	NK cells	CD19- CD3e- NK1.1+ (CD11b+)	6
	proinflammatory monocytes/macrophages	CD19- CD3e- NK1.1- CD11b+ Ly6G- Ly6C+	6
	MHCII+ proinfl. MoMacs	CD19- CD3e- NK1.1- CD11b+ Ly6G- Ly6C+ MHCII+	6
	pDCs	CD19- CD3e- NK1.1- CD11c+ CD11b- MHCII+ B220+ BST-2+	6

Peritoneal cavity	**B cells**	CD19+	8
	B-2 cells	CD19+ B220(high)	8
	B-1 cells	CD19+ B220(low/int)	8
	B-1a cells	CD19+ B220(low/int) CD5+ (CD11b+)	8
	B-1a cells (B-1 independent)	CD19+ CD5+ (CD11b+)	8
	B-1b cells	CD19+ B220(low/int) CD5- (CD11b+)	8
	dead B cells	FVD eFluor780+ CD19+	8
	T cells	CD19- CD5+	8
	CD4+ T cells	CD19- CD5+ CD4+ CD8a-	8
	CD8+ T cells	CD19- CD5+ CD4- CD8a+	8
	double-negative T cells	CD19- CD5+ CD4- CD8a-	8
	neutrophils	CD19- CD5- NK1.1- CD11b+ Ly6G+	8
	proinflammatory monocytes/macrophages	CD19- CD5- NK1.1- CD11b+ Ly6G- Ly6C+	8
	MHCII+ proinfl. MoMacs	CD19- CD5- NK1.1- CD11b+ Ly6G- Ly6C+ MHCII+	8
Blood	**B cells**	CD19+	-
	early B cells	CD19+ AA4.1+ IgM-	-
	mature B cells	CD19+ AA4.1- IgM+ IgD+	-
	AA4.1+ myeloid cells	AA4.1+ CD11b+	-
	neutrophils	CD19- CD3e- NK1.1- CD11b+ Ly6G+	similar to 1
	MHCII+ proinfl. MoMacs	CD19- CD3e- NK1.1- CD11b+ Ly6G- Ly6C+ MHCII+	similar to 1

Supplementary Table 2: Identified immune cell populations in the BM, PerC, and blood of mice.

Acknowledgements

We thank Julia Mock, Nicole Küpper, Sonja Schavier and Saskia van Essen-van Dorresteijn for technical assistance.

This work was supported by the Jürgen Manchot Foundation (Molecules of Infection IV [MOI IV]).

Computational support of the Zentrum für Informations- und Medientechnologie, especially the HPC team (High Performance Computing) at the Heinrich-Heine University is acknowledged.

We also acknowledge funding by the German Federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung; Netzwerk Universitätsmedizin, GenSurv/MolTraX), award number 01KX2021.

Authorship

Contribution: M.H. performed and analyzed all experiments, except for Fig. 3 & 6 and Suppl. Fig. 9, 10 and 12, with support from U.R.S. J.P. analyzed and visualized RNA-seq data sets as shown in Fig. 3 and Suppl. Fig. 9 & 10. P.P. and K.K. performed the 3' RNA sequencing. R.E.T. performed the histological analysis as shown in Fig. 6 and Suppl. Fig. 12. with support from A.dB. K.Pracht and H-M.J. provided guidance on the identification of B cell subsets using flow cytometry and on all B cell-related topics. M.H., U.R.S., and K.Pfeffer designed the study and wrote the manuscript with input from D.D.

We declare no competing interests.

Literature

1. Wimmer, N., Huber, B., Barabas, N., Rohrl, J., Pfeffer, K., and Hehlgans, T. (2012). *Lymphotoxin beta receptor activation on macrophages induces cross-tolerance to TLR4 and TLR9 ligands*. J Immunol ***188***, 3426-3433. 10.4049/jimmunol.1103324.
2. Wimmer, N., Huber, B., Wege, A.K., Barabas, N., Rohrl, J., Pfeffer, K., and Hehlgans, T. (2012). *Lymphotoxin-beta receptor activation on macrophages ameliorates acute DSS-induced intestinal inflammation in a TRIM30alpha-dependent manner*. Mol Immunol ***51***, 128-135. 10.1016/j.molimm.2012.02.118.
3. Riffelmacher, T., Giles, D.A., Zahner, S., Dicker, M., Andreyev, A.Y., McArdle, S., Perez-Jeldres, T., van der Gracht, E., Murray, M.P., Hartmann, N., Tumanov, A.V., and Kronenberg, M. (2021). *Metabolic activation and colitis pathogenesis is prevented by lymphotoxin beta receptor expression in neutrophils*. Mucosal Immunol ***14***, 679-690. 10.1038/s41385-021-00378-7.
4. Wang, Y.G., Kim, K.D., Wang, J., Yu, P., and Fu, Y.X. (2005). *Stimulating lymphotoxin beta receptor on the dendritic cells is critical for their homeostasis and expansion*. J Immunol ***175***, 6997-7002. 10.4049/jimmunol.175.10.6997.
5. Kabashima, K., Banks, T.A., Ansel, K.M., Lu, T.T., Ware, C.F., and Cyster, J.G. (2005). *Intrinsic lymphotoxin-beta receptor requirement for homeostasis of lymphoid tissue dendritic cells*. Immunity ***22***, 439-450. 10.1016/j.immuni.2005.02.007.
6. De Trez, C. (2012). *Lymphotoxin-beta receptor expression and its related signaling pathways govern dendritic cell homeostasis and function*. Immunobiology ***217***, 1250-1258. 10.1016/j.imbio.2012.06.010.
7. Ware, C.F. (2005). *Network communications: lymphotoxins, LIGHT, and TNF*. Annu Rev Immunol ***23***, 787-819. 10.1146/annurev.immunol.23.021704.115719.
8. Hehlgans, T., and Pfeffer, K. (2005). *The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games*. Immunology ***115***, 1-20. 10.1111/j.1365-2567.2005.02143.x.
9. Ward-Kavanagh, L.K., Lin, W.W., Sedy, J.R., and Ware, C.F. (2016). *The TNF Receptor Superfamily in Co-stimulating and Co-inhibitory Responses*. Immunity ***44***, 1005-1019. 10.1016/j.immuni.2016.04.019.

10. Dejardin, E., Droin, N.M., Delhase, M., Haas, E., Cao, Y., Makris, C., Li, Z.W., Karin, M., Ware, C.F., and Green, D.R. (2002). *The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways*. Immunity ***17***, 525-535. 10.1016/s1074-7613(02)00423-5.

11. Futterer, A., Mink, K., Luz, A., Kosco-Vilbois, M.H., and Pfeffer, K. (1998). *The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues*. Immunity ***9***, 59-70.

12. Shou, Y., Koroleva, E., Spencer, C.M., Shein, S.A., Korchagina, A.A., Yusoof, K.A., Parthasarathy, R., Leadbetter, E.A., Akopian, A.N., Munoz, A.R., and Tumanov, A.V. (2021). *Redefining the Role of Lymphotoxin Beta Receptor in the Maintenance of Lymphoid Organs and Immune Cell Homeostasis in Adulthood*. Front Immunol ***12***, 712632. 10.3389/fimmu.2021.712632.

13. Wu, Q., Wang, Y., Wang, J., Hedgeman, E.O., Browning, J.L., and Fu, Y.X. (1999). *The requirement of membrane lymphotoxin for the presence of dendritic cells in lymphoid tissues*. J Exp Med ***190***, 629-638. 10.1084/jem.190.5.629.

14. Elewaut, D., Brossay, L., Santee, S.M., Naidenko, O.V., Burdin, N., De Winter, H., Matsuda, J., Ware, C.F., Cheroutre, H., and Kronenberg, M. (2000). *Membrane lymphotoxin is required for the development of different subpopulations of NK T cells*. J Immunol ***165***, 671-679. 10.4049/jimmunol.165.2.671.

15. Franki, A.S., Van Beneden, K., Dewint, P., Hammond, K.J., Lambrecht, S., Leclercq, G., Kronenberg, M., Deforce, D., and Elewaut, D. (2006). *A unique lymphotoxin alphabeta-dependent pathway regulates thymic emigration of Valpha14 invariant natural killer T cells*. Proc Natl Acad Sci U S A ***103***, 9160-9165. 10.1073/pnas.0508892103.

16. Iizuka, K., Chaplin, D.D., Wang, Y., Wu, Q., Pegg, L.E., Yokoyama, W.M., and Fu, Y.X. (1999). *Requirement for membrane lymphotoxin in natural killer cell development*. Proc Natl Acad Sci U S A ***96***, 6336-6340. 10.1073/pnas.96.11.6336.

17. Tersteegen, A., Sorg, U.R., Virgen-Slane, R., Helle, M., Petzsch, P., Dunay, I.R., Kohrer, K., Degrandi, D., Ware, C.F., and Pfeffer, K. (2021). *Lymphotoxin beta Receptor: a Crucial Role in Innate and Adaptive Immune Responses against Toxoplasma gondii*. Infect Immun ***89***. 10.1128/IAI.00026-21.

18. Borelli, A., and Irla, M. (2021). *Lymphotoxin: from the physiology to the regeneration of the thymic function*. Cell Death Differ ***28***, 2305-2314. 10.1038/s41418-021-00834-8.

19. Fu, Y.X., Huang, G., Wang, Y., and Chaplin, D.D. (1998). *B lymphocytes induce the formation of follicular dendritic cell clusters in a lymphotoxin alpha-dependent fashion*. J Exp Med ***187***, 1009-1018. 10.1084/jem.187.7.1009.

20. Ngo, V.N., Korner, H., Gunn, M.D., Schmidt, K.N., Riminton, D.S., Cooper, M.D., Browning, J.L., Sedgwick, J.D., and Cyster, J.G. (1999). *Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen*. J Exp Med ***189***, 403-412. 10.1084/jem.189.2.403.

21. Vu, F., Dianzani, U., Ware, C.F., Mak, T., and Gommerman, J.L. (2008). *ICOS, CD40, and lymphotoxin beta receptors signal sequentially and interdependently to initiate a germinal center reaction*. J Immunol ***180***, 2284-2293. 10.4049/jimmunol.180.4.2284.

22. Zindl, C.L., Kim, T.H., Zeng, M., Archambault, A.S., Grayson, M.H., Choi, K., Schreiber, R.D., and Chaplin, D.D. (2009). *The lymphotoxin LTalpha(1)beta(2) controls postnatal and adult spleen marginal sinus vascular structure and function*. Immunity ***30***, 408-420. 10.1016/j.immuni.2009.01.010.

23. Myers, R.C., King, R.G., Carter, R.H., and Justement, L.B. (2013). *Lymphotoxin alpha1beta2 expression on B cells is required for follicular dendritic cell activation during the germinal center response*. Eur J Immunol ***43***, 348-359. 10.1002/eji.201242471.

24. Camara, A., Lavanant, A.C., Abe, J., Desforges, H.L., Alexandre, Y.O., Girardi, E., Igamberdieva, Z., Asano, K., Tanaka, M., Hehlgans, T., Pfeffer, K., Pfeffer, S., Mueller, S.N., Stein, J.V., and Mueller, C.G. (2022). *CD169(+) macrophages in lymph node and spleen critically depend on dual RANK and LTbetaR signaling*. Proc Natl Acad Sci U S A ***119***. 10.1073/pnas.2108540119.

25. Banks, T.A., Rickert, S., and Ware, C.F. (2006). *Restoring immune defenses via lymphotoxin signaling: lessons from cytomegalovirus*. Immunol Res ***34***, 243-254. 10.1385/IR:34:3:243.

26. Behnke, K., Sorg, U.R., Gabbert, H.E., and Pfeffer, K. (2017). *The Lymphotoxin beta Receptor Is Essential for Upregulation of IFN-Induced Guanylate-Binding Proteins and Survival after Toxoplasma gondii Infection*. Mediators Inflamm ***2017***, 7375818. 10.1155/2017/7375818.

27. Ehlers, S., Holscher, C., Scheu, S., Tertilt, C., Hehlgans, T., Suwinski, J., Endres, R., and Pfeffer, K. (2003). *The lymphotoxin beta receptor is critically involved in controlling infections with the intracellular pathogens Mycobacterium tuberculosis and Listeria monocytogenes*. J Immunol ***170***, 5210-5218.

28. Jin, L., Guo, X., Shen, C., Hao, X., Sun, P., Li, P., Xu, T., Hu, C., Rose, O., Zhou, H., Yang, M., Qin, C.F., Guo, J., Peng, H., Zhu, M., Cheng, G., Qi, X., and Lai, R. (2018). *Salivary factor LTRIN from Aedes aegypti facilitates the transmission of Zika virus by interfering with the lymphotoxin-beta receptor*. Nat Immunol ***19***, 342-353. 10.1038/s41590-018-0063-9.

29. Puglielli, M.T., Browning, J.L., Brewer, A.W., Schreiber, R.D., Shieh, W.J., Altman, J.D., Oldstone, M.B., Zaki, S.R., and Ahmed, R. (1999). *Reversal of virus-induced systemic shock and respiratory failure by blockade of the lymphotoxin pathway*. Nat Med ***5***, 1370-1374. 10.1038/70938.

30. Spahn, T.W., Maaser, C., Eckmann, L., Heidemann, J., Lugering, A., Newberry, R., Domschke, W., Herbst, H., and Kucharzik, T. (2004). *The lymphotoxin-beta receptor is critical for control of murine Citrobacter rodentium-induced colitis*. Gastroenterology ***127***, 1463-1473. 10.1053/j.gastro.2004.08.022.

31. Boehm, T., Scheu, S., Pfeffer, K., and Bleul, C.C. (2003). *Thymic medullary epithelial cell differentiation, thymocyte emigration, and the control of autoimmunity require lympho-epithelial cross talk via LTbetaR*. J Exp Med ***198***, 757-769. 10.1084/jem.20030794.

32. Cosway, E.J., Lucas, B., James, K.D., Parnell, S.M., Carvalho-Gaspar, M., White, A.J., Tumanov, A.V., Jenkinson, W.E., and Anderson, G. (2017). *Redefining thymus medulla specialization for central tolerance*. J Exp Med ***214***, 3183-3195. 10.1084/jem.20171000.

33. Fernandes, M.T., Dejardin, E., and dos Santos, N.R. (2016). *Context-dependent roles for lymphotoxin-beta receptor signaling in cancer development*. Biochim Biophys Acta ***1865***, 204-219. 10.1016/j.bbcan.2016.02.005.

34. Wolf, M.J., Seleznik, G.M., Zeller, N., and Heikenwalder, M. (2010). *The unexpected role of lymphotoxin beta receptor signaling in carcinogenesis: from lymphoid tissue formation to liver and prostate cancer development*. Oncogene ***29***, 5006-5018. 10.1038/onc.2010.260.

35. Seleznik, G., Seeger, H., Bauer, J., Fu, K., Czerkowicz, J., Papandile, A., Poreci, U., Rabah, D., Ranger, A., Cohen, C.D., Lindenmeyer, M., Chen, J., Edenhofer, I., Anders, H.J., Lech, M., Wuthrich, R.P., Ruddle, N.H., Moeller, M.J., Kozakowski, N., Regele, H., Browning, J.L., Heikenwalder, M., and Segerer, S. (2016). *The lymphotoxin beta receptor is a potential therapeutic target in renal inflammation*. Kidney Int ***89***, 113-126. 10.1038/ki.2015.280.

36. Zhang, G., Feizi, N., Zhao, D., Halesha, L., Williams, A.L., Randhawa, P.S., Abou-Daya, K.I., and Oberbarnscheidt, M.H. (2024). *Lymphotoxin beta receptor and tertiary lymphoid organs shape acute and chronic allograft rejection*. JCI Insight. 10.1172/jci.insight.177555.

37. Robert-Gangneux, F., and Darde, M.L. (2012). *Epidemiology of and diagnostic strategies for toxoplasmosis*. Clin Microbiol Rev ***25***, 264-296. 10.1128/CMR.05013-11.

38. English, E.D., and Striepen, B. (2019). *The cat is out of the bag: How parasites know their hosts*. PLoS Biol ***17***, e3000446. 10.1371/journal.pbio.3000446.

39. Daher, D., Shaghlil, A., Sobh, E., Hamie, M., Hassan, M.E., Moumneh, M.B., Itani, S., El Hajj, R., Tawk, L., El Sabban, M., and El Hajj, H. (2021). *Comprehensive Overview of Toxoplasma gondii-Induced and Associated Diseases*. Pathogens ***10***. 10.3390/pathogens10111351.

40. Zhang, Y., Li, D., Lu, S., and Zheng, B. (2022). *Toxoplasmosis vaccines: what we have and where to go?* NPJ Vaccines ***7***, 131. 10.1038/s41541-022-00563-0.

41. Khan, I.A., and Moretto, M. (2022). *Immune responses to Toxoplasma gondii*. Curr Opin Immunol ***77***, 102226. 10.1016/j.coi.2022.102226.

42. Iwasaki, A., and Medzhitov, R. (2010). *Regulation of adaptive immunity by the innate immune system*. Science ***327***, 291-295. 10.1126/science.1183021.

43. Sasai, M., and Yamamoto, M. (2019). *Innate, adaptive, and cell-autonomous immunity against Toxoplasma gondii infection*. Exp Mol Med ***51***, 1-10. 10.1038/s12276-019-0353-9.

44. Yarovinsky, F. (2014). *Innate immunity to Toxoplasma gondii infection*. Nat Rev Immunol ***14***, 109-121. 10.1038/nri3598.

45. Gazzinelli, R.T., Hieny, S., Wynn, T.A., Wolf, S., and Sher, A. (1993). *Interleukin 12 is required for the T-lymphocyte-independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts*. Proc Natl Acad Sci U S A ***90***, 6115-6119. 10.1073/pnas.90.13.6115.

46. Hunter, C.A., Subauste, C.S., Van Cleave, V.H., and Remington, J.S. (1994). *Production of gamma interferon by natural killer cells from Toxoplasma gondii-infected SCID mice: regulation by interleukin-10, interleukin-12, and tumor necrosis factor alpha*. Infect Immun ***62***, 2818-2824. 10.1128/iai.62.7.2818-2824.1994.

47. Hou, B., Benson, A., Kuzmich, L., DeFranco, A.L., and Yarovinsky, F. (2011). *Critical coordination of innate immune defense against Toxoplasma gondii by dendritic cells responding via their Toll-like receptors*. Proc Natl Acad Sci U S A ***108***, 278-283. 10.1073/pnas.1011549108.

48. Wilson, D.C., Matthews, S., and Yap, G.S. (2008). *IL-12 signaling drives CD8+ T cell IFN-gamma production and differentiation of KLRG1+ effector subpopulations during Toxoplasma gondii Infection*. J Immunol ***180***, 5935-5945. 10.4049/jimmunol.180.9.5935.

49. Yap, G., Pesin, M., and Sher, A. (2000). *Cutting edge: IL-12 is required for the maintenance of IFN-gamma production in T cells mediating chronic resistance to the intracellular pathogen, Toxoplasma gondii*. J Immunol ***165***, 628-631. 10.4049/jimmunol.165.2.628.

50. Spits, H., Bernink, J.H., and Lanier, L. (2016). *NK cells and type 1 innate lymphoid cells: partners in host defense*. Nat Immunol ***17***, 758-764. 10.1038/ni.3482.

51. Suzuki, Y., Orellana, M.A., Schreiber, R.D., and Remington, J.S. (1988). *Interferon-gamma: the major mediator of resistance against Toxoplasma gondii*. Science ***240***, 516-518. 10.1126/science.3128869.

52. Yap, G.S., and Sher, A. (1999). *Cell-mediated immunity to Toxoplasma gondii: initiation, regulation and effector function*. Immunobiology ***201***, 240-247. 10.1016/S0171-2985(99)80064-3.

53. Haldar, A.K., Saka, H.A., Piro, A.S., Dunn, J.D., Henry, S.C., Taylor, G.A., Frickel, E.M., Valdivia, R.H., and Coers, J. (2013). *IRG and GBP host resistance factors target aberrant, "non-self" vacuoles characterized by the missing of "self" IRGM proteins*. PLoS Pathog ***9***, e1003414. 10.1371/journal.ppat.1003414.

54. Henry, S.C., Daniell, X.G., Burroughs, A.R., Indaram, M., Howell, D.N., Coers, J., Starnbach, M.N., Hunn, J.P., Howard, J.C., Feng, C.G., Sher, A., and Taylor, G.A. (2009). *Balance of Irgm protein activities determines IFN-gamma-induced host defense*. J Leukoc Biol ***85***, 877-885. 10.1189/jlb.1008599.

55. Hunn, J.P., Koenen-Waisman, S., Papic, N., Schroeder, N., Pawlowski, N., Lange, R., Kaiser, F., Zerrahn, J., Martens, S., and Howard, J.C. (2008). *Regulatory interactions between IRG resistance GTPases in the cellular response to Toxoplasma gondii*. EMBO J ***27***, 2495-2509. 10.1038/emboj.2008.176.

56. Khaminets, A., Hunn, J.P., Konen-Waisman, S., Zhao, Y.O., Preukschat, D., Coers, J., Boyle, J.P., Ong, Y.C., Boothroyd, J.C., Reichmann, G., and Howard, J.C. (2010). *Coordinated loading of IRG resistance GTPases on to the Toxoplasma gondii parasitophorous vacuole*. Cell Microbiol ***12***, 939-961. 10.1111/j.1462-5822.2010.01443.x.

57. Martens, S., Parvanova, I., Zerrahn, J., Griffiths, G., Schell, G., Reichmann, G., and Howard, J.C. (2005). *Disruption of Toxoplasma gondii parasitophorous vacuoles by the mouse p47-resistance GTPases*. PLoS Pathog ***1***, e24. 10.1371/journal.ppat.0010024.

58. Degrandi, D., Kravets, E., Konermann, C., Beuter-Gunia, C., Klumpers, V., Lahme, S., Rasch, E., Mausberg, A.K., Beer-Hammer, S., and Pfeffer, K. (2013). *Murine guanylate binding protein 2 (mGBP2) controls Toxoplasma gondii replication*. Proc Natl Acad Sci U S A ***110***, 294-299. 10.1073/pnas.1205635110.

59. Kravets, E., Degrandi, D., Weidtkamp-Peters, S., Ries, B., Konermann, C., Felekyan, S., Dargazanli, J.M., Praefcke, G.J., Seidel, C.A., Schmitt, L., Smits, S.H., and Pfeffer, K. (2012). *The GTPase activity of murine guanylate-binding protein 2 (mGBP2) controls the intracellular localization and recruitment to the parasitophorous vacuole of Toxoplasma gondii*. J Biol Chem ***287***, 27452-27466. 10.1074/jbc.M112.379636.

60. Selleck, E.M., Fentress, S.J., Beatty, W.L., Degrandi, D., Pfeffer, K., Virgin, H.W.t., Macmicking, J.D., and Sibley, L.D. (2013). *Guanylate-binding protein 1 (Gbp1) contributes to cell-autonomous immunity against Toxoplasma gondii*. PLoS Pathog ***9***, e1003320. 10.1371/journal.ppat.1003320.

61. Steffens, N., Beuter-Gunia, C., Kravets, E., Reich, A., Legewie, L., Pfeffer, K., and Degrandi, D. (2020). *Essential Role of mGBP7 for Survival of Toxoplasma gondii Infection*. mBio ***11***. 10.1128/mBio.02993-19.

62. Yamamoto, M., Okuyama, M., Ma, J.S., Kimura, T., Kamiyama, N., Saiga, H., Ohshima, J., Sasai, M., Kayama, H., Okamoto, T., Huang, D.C., Soldati-Favre, D., Horie, K., Takeda, J., and Takeda, K. (2012). *A cluster of interferon-gamma-inducible p65 GTPases plays a critical role in host defense against Toxoplasma gondii*. Immunity ***37***, 302-313. 10.1016/j.immuni.2012.06.009.

63. El Bissati, K., Krishack, P.A., Zhou, Y., Weber, C.R., Lykins, J., Jankovic, D., Edelblum, K.L., Fraczek, L., Grover, H., Chentoufi, A.A., Singh, G., Reardon, C., Dubey, J.P., Reed, S., Alexander, J., Sidney, J., Sette, A., Shastri, N., and McLeod, R. (2023). *CD4(+) T Cell Responses to Toxoplasma gondii Are a Double-Edged Sword*. Vaccines (Basel) ***11***. 10.3390/vaccines11091485.

64. Gazzinelli, R.T., Wysocka, M., Hieny, S., Scharton-Kersten, T., Cheever, A., Kuhn, R., Muller, W., Trinchieri, G., and Sher, A. (1996). *In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha*. J Immunol ***157***, 798-805.

65. Lopez-Yglesias, A.H., Burger, E., Araujo, A., Martin, A.T., and Yarovinsky, F. (2018). *T-bet-independent Th1 response induces intestinal immunopathology during Toxoplasma gondii infection*. Mucosal Immunol ***11***, 921-931. 10.1038/mi.2017.102.

66. Olguin, J.E., Fernandez, J., Salinas, N., Juarez, I., Rodriguez-Sosa, M., Campuzano, J., Castellanos, C., and Saavedra, R. (2015). *Adoptive transfer of CD4(+)Foxp3(+) regulatory T cells to C57BL/6J mice during acute infection with Toxoplasma gondii down modulates the exacerbated Th1 immune response*. Microbes Infect ***17***, 586-595. 10.1016/j.micinf.2015.04.002.

67. Saraav, I., Cervantes-Barragan, L., Olias, P., Fu, Y., Wang, Q., Wang, L., Wang, Y., Mack, M., Baldridge, M.T., Stappenbeck, T., Colonna, M., and Sibley, L.D. (2021). *Chronic Toxoplasma gondii infection enhances susceptibility to colitis*. Proc Natl Acad Sci U S A ***118***. 10.1073/pnas.2106730118.

68. Warunek, J., Jin, R.M., Blair, S.J., Garis, M., Marzullo, B., and Wohlfert, E.A. (2021). *Tbet Expression by Regulatory T Cells Is Needed to Protect against Th1-Mediated Immunopathology during Toxoplasma Infection in Mice*. Immunohorizons ***5***, 931-943. 10.4049/immunohorizons.2100080.

69. Zhang, Z.X., Yang, L., Young, K.J., DuTemple, B., and Zhang, L. (2000). *Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression*. Nat Med ***6***, 782-789. 10.1038/77513.

70. Merims, S., Li, X., Joe, B., Dokouhaki, P., Han, M., Childs, R.W., Wang, Z.Y., Gupta, V., Minden, M.D., and Zhang, L. (2011). *Anti-leukemia effect of ex vivo expanded DNT cells from AML patients: a potential novel autologous T-cell adoptive immunotherapy*. Leukemia ***25***, 1415-1422. 10.1038/leu.2011.99.

71. Sykes, M. (1990). *Unusual T cell populations in adult murine bone marrow. Prevalence of CD3+CD4-CD8- and alpha beta TCR+NK1.1+ cells*. J Immunol ***145***, 3209-3215.

72. Wu, Z., Zheng, Y., Sheng, J., Han, Y., Yang, Y., Pan, H., and Yao, J. (2022). *CD3(+)CD4(-)CD8(-) (Double-Negative) T Cells in Inflammation, Immune Disorders and Cancer*. Front Immunol ***13***, 816005. 10.3389/fimmu.2022.816005.

73. Hu, Z., Yang, M., Chen, H., He, C., Lin, Z., Yang, X., Li, H., Shen, W., Lu, D., and Xu, X. (2023). *Double-negative T cells: a promising avenue of adoptive cell therapy in transplant oncology*. J Zhejiang Univ Sci B ***24***, 387-396. 10.1631/jzus.B2200528.

74. Li, H., and Tsokos, G.C. (2021). *Double-negative T cells in autoimmune diseases*. Curr Opin Rheumatol ***33***, 163-172. 10.1097/BOR.0000000000000778.

75. Martina, M.N., Noel, S., Saxena, A., Rabb, H., and Hamad, A.R. (2015). *Double negative (DN) alphabeta T cells: misperception and overdue recognition*. Immunol Cell Biol ***93***, 305-310. 10.1038/icb.2014.99.

76. Hammouda, N.A., Abo el-Naga, I., Hussein, E.D., and Rashwan, E.A. (1995). *Opsonization and intracellular killing of Toxoplasma gondii by human mononuclear phagocytes*. J Egypt Soc Parasitol ***25***, 11-17.

77. Vercammen, M., Scorza, T., El Bouhdidi, A., Van Beeck, K., Carlier, Y., Dubremetz, J.F., and Verschueren, H. (1999). *Opsonization of Toxoplasma gondii tachyzoites with nonspecific immunoglobulins promotes their phagocytosis by macrophages and inhibits their proliferation in nonphagocytic cells in tissue culture*. Parasite Immunol ***21***, 555-563. 10.1046/j.1365-3024.1999.00256.x.

78. Kang, H., Remington, J.S., and Suzuki, Y. (2000). *Decreased resistance of B cell-deficient mice to infection with Toxoplasma gondii despite unimpaired expression of IFN-gamma, TNF-alpha, and inducible nitric oxide synthase*. J Immunol ***164***, 2629-2634. 10.4049/jimmunol.164.5.2629.

79. Sayles, P.C., Gibson, G.W., and Johnson, L.L. (2000). *B cells are essential for vaccination-induced resistance to virulent Toxoplasma gondii*. Infect Immun ***68***, 1026-1033. 10.1128/IAI.68.3.1026-1033.2000.

80. Johnson, L.L., and Sayles, P.C. (2002). *Deficient humoral responses underlie susceptibility to Toxoplasma gondii in CD4-deficient mice*. Infect Immun ***70***, 185-191. 10.1128/IAI.70.1.185-191.2002.

81. Chen, M., Mun, H.S., Piao, L.X., Aosai, F., Norose, K., Mohamed, R.M., Belal, U.S., Fang, H., Ahmed, A.K., Kang, H.K., Matsuzaki, G., Kitamura, D., and Yano, A. (2003). *Induction of protective immunity by primed B-1 cells in Toxoplasma gondii -infected B cell-deficient mice*. Microbiol Immunol ***47***, 997-1003. 10.1111/j.1348-0421.2003.tb03460.x.

82. Johnson, L.L., Lanthier, P., Hoffman, J., and Chen, W. (2004). *Vaccination protects B cell-deficient mice against an oral challenge with mildly virulent Toxoplasma gondii*. Vaccine ***22***, 4054-4061. 10.1016/j.vaccine.2004.03.056.

83. Couper, K.N., Roberts, C.W., Brombacher, F., Alexander, J., and Johnson, L.L. (2005). *Toxoplasma gondii-specific immunoglobulin M limits parasite dissemination by preventing host cell invasion*. Infect Immun ***73***, 8060-8068. 10.1128/IAI.73.12.8060-8068.2005.

84. Souza, S.P., Splitt, S.D., Sanchez-Arcila, J.C., Alvarez, J.A., Wilson, J.N., Wizzard, S., Luo, Z., Baumgarth, N., and Jensen, K.D.C. (2021). *Genetic mapping reveals Nfkbid as a central regulator of humoral immunity to Toxoplasma gondii*. PLoS Pathog ***17***, e1010081. 10.1371/journal.ppat.1010081.

85. Kantor, A.B., Stall, A.M., Adams, S., Herzenberg, L.A., and Herzenberg, L.A. (1992). *Differential development of progenitor activity for three B-cell lineages*. Proc Natl Acad Sci U S A ***89***, 3320-3324. 10.1073/pnas.89.8.3320.

86. Cariappa, A., Chase, C., Liu, H., Russell, P., and Pillai, S. (2007). *Naive recirculating B cells mature simultaneously in the spleen and bone marrow*. Blood ***109***, 2339-2345. 10.1182/blood-2006-05-021089.

87. Shahaf, G., Zisman-Rozen, S., Benhamou, D., Melamed, D., and Mehr, R. (2016). *B Cell Development in the Bone Marrow Is Regulated by Homeostatic Feedback Exerted by Mature B Cells*. Front Immunol ***7***, 77. 10.3389/fimmu.2016.00077.

88. Baumgarth, N. (2011). *The double life of a B-1 cell: self-reactivity selects for protective effector functions*. Nat Rev Immunol ***11***, 34-46. 10.1038/nri2901.

89. Kantor, A.B., Merrill, C.E., Herzenberg, L.A., and Hillson, J.L. (1997). *An unbiased analysis of V(H)-D-J(H) sequences from B-1a, B-1b, and conventional B cells*. J Immunol ***158***, 1175-1186.

90. Martin, F., Oliver, A.M., and Kearney, J.F. (2001). *Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens*. Immunity ***14***, 617-629. 10.1016/s1074-7613(01)00129-7.

91. Haas, K.M., Poe, J.C., Steeber, D.A., and Tedder, T.F. (2005). *B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae*. Immunity ***23***, 7-18. 10.1016/j.immuni.2005.04.011.

92. Choi, Y.S., Dieter, J.A., Rothaeusler, K., Luo, Z., and Baumgarth, N. (2012). *B-1 cells in the bone marrow are a significant source of natural IgM*. Eur J Immunol ***42***, 120-129. 10.1002/eji.201141890.

93. Holodick, N.E., Rodriguez-Zhurbenko, N., and Hernandez, A.M. (2017). *Defining Natural Antibodies*. Front Immunol ***8***, 872. 10.3389/fimmu.2017.00872.

94. Smith, F.L., and Baumgarth, N. (2019). *B-1 cell responses to infections*. Curr Opin Immunol ***57***, 23-31. 10.1016/j.coi.2018.12.001.

95. Chen, M., Aosai, F., Mun, H.S., Norose, K., Hata, H., and Yano, A. (2000). *Anti-HSP70 autoantibody formation by B-1 cells in Toxoplasma gondii-infected mice*. Infect Immun ***68***, 4893-4899. 10.1128/IAI.68.9.4893-4899.2000.

96. Chen, M., Aosai, F., Norose, K., Mun, H.S., and Yano, A. (2003). *The role of anti-HSP70 autoantibody-forming V(H)1-J(H)1 B-1 cells in Toxoplasma gondii-infected mice*. Int Immunol ***15***, 39-47. 10.1093/intimm/dxg004.

97. Masihi, K.N., and Werner, H. (1977). *Suppression and enhancement of humoral immune response to Toxoplasma gondii by passive antibody*. Experientia ***33***, 1586-1587. 10.1007/BF01934012.

98. Strickland, G.T., and Sayles, P.C. (1977). *Depressed antibody responses to a thymus-dependent antigen in toxoplasmosis*. Infect Immun ***15***, 184-190. 10.1128/iai.15.1.184-190.1977.

99. Kobayashi, A., and Suzuki, Y. (1987). *Suppression of antibody responses by Toxoplasma infection in mice*. Zentralbl Bakteriol Mikrobiol Hyg A ***264***, 312-318. 10.1016/s0176-6724(87)80049-4.

100. Amezcua Vesely, M.C., Bermejo, D.A., Montes, C.L., Acosta-Rodriguez, E.V., and Gruppi, A. (2012). *B-Cell Response during Protozoan Parasite Infections*. J Parasitol Res ***2012***, 362131. 10.1155/2012/362131.

101. Lim, V.Y., Zehentmeier, S., Fistonich, C., and Pereira, J.P. (2017). *A Chemoattractant-Guided Walk Through Lymphopoiesis: From Hematopoietic Stem Cells to Mature B Lymphocytes*. Adv Immunol ***134***, 47-88. 10.1016/bs.ai.2017.02.001.

102. Ueda, Y., Yang, K., Foster, S.J., Kondo, M., and Kelsoe, G. (2004). *Inflammation controls B lymphopoiesis by regulating chemokine CXCL12 expression*. J Exp Med ***199***, 47-58. 10.1084/jem.20031104.

103. Pracht, K., Meinzinger, J., Daum, P., Schulz, S.R., Reimer, D., Hauke, M., Roth, E., Mielenz, D., Berek, C., Corte-Real, J., Jack, H.M., and Schuh, W. (2017). *A new staining protocol for detection of murine antibody-secreting plasma cell subsets by flow cytometry*. Eur J Immunol ***47***, 1389-1392. 10.1002/eji.201747019.

104. Kim, T.J., Upadhyay, V., Kumar, V., Lee, K.M., and Fu, Y.X. (2014). *Innate lymphoid cells facilitate NK cell development through a lymphotoxin-mediated stromal microenvironment*. J Exp Med ***211***, 1421-1431. 10.1084/jem.20131501.

105. Summers-DeLuca, L.E., McCarthy, D.D., Cosovic, B., Ward, L.A., Lo, C.C., Scheu, S., Pfeffer, K., and Gommerman, J.L. (2007). *Expression of lymphotoxin-alphabeta on antigen-specific T cells is required for DC function*. J Exp Med ***204***, 1071-1081. 10.1084/jem.20061968.

106. Wang, G., Abadia-Molina, A.C., Berger, S.B., Romero, X., O'Keeffe, M.S., Rojas-Barros, D.I., Aleman, M., Liao, G., Maganto-Garcia, E., Fresno, M., Wang, N., Detre, C., and Terhorst, C. (2012). *Cutting edge: Slamf8 is a negative regulator of Nox2 activity in macrophages*. J Immunol ***188***, 5829-5832. 10.4049/jimmunol.1102620.

107. Aziz, M., Holodick, N.E., Rothstein, T.L., and Wang, P. (2015). *The role of B-1 cells in inflammation*. Immunol Res ***63***, 153-166. 10.1007/s12026-015-8708-3.

108. Baumgarth, N. (2016). *B-1 Cell Heterogeneity and the Regulation of Natural and Antigen-Induced IgM Production*. Front Immunol ***7***, 324. 10.3389/fimmu.2016.00324.

109. Ansel, K.M., Harris, R.B., and Cyster, J.G. (2002). *CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity*. Immunity ***16***, 67-76. 10.1016/s1074-7613(01)00257-6.

110. Ha, S.A., Tsuji, M., Suzuki, K., Meek, B., Yasuda, N., Kaisho, T., and Fagarasan, S. (2006). *Regulation of B1 cell migration by signals through Toll-like receptors*. J Exp Med ***203***, 2541-2550. 10.1084/jem.20061041.

111. Bockstal, V., Guirnalda, P., Caljon, G., Goenka, R., Telfer, J.C., Frenkel, D., Radwanska, M., Magez, S., and Black, S.J. (2011). *T. brucei infection reduces B lymphopoiesis in bone marrow*

and truncates compensatory splenic lymphopoiesis through transitional B-cell apoptosis. PLoS Pathog ***7***, e1002089. 10.1371/journal.ppat.1002089.

112. Bockstal, V., Geurts, N., and Magez, S. (2011). *Acute Disruption of Bone Marrow B Lymphopoiesis and Apoptosis of Transitional and Marginal Zone B Cells in the Spleen following a Blood-Stage Plasmodium chabaudi Infection in Mice*. J Parasitol Res ***2011***, 534697. 10.1155/2011/534697.

113. Boada-Romero, E., Martinez, J., Heckmann, B.L., and Green, D.R. (2020). *The clearance of dead cells by efferocytosis*. Nat Rev Mol Cell Biol ***21***, 398-414. 10.1038/s41580-020-0232-1.

114. Hochreiter-Hufford, A., and Ravichandran, K.S. (2013). *Clearing the dead: apoptotic cell sensing, recognition, engulfment, and digestion*. Cold Spring Harb Perspect Biol ***5***, a008748. 10.1101/cshperspect.a008748.

115. Mammari, N., Halabi, M.A., Yaacoub, S., Chlala, H., Darde, M.L., and Courtioux, B. (2019). *Toxoplasma gondii Modulates the Host Cell Responses: An Overview of Apoptosis Pathways*. Biomed Res Int ***2019***, 6152489. 10.1155/2019/6152489.

116. Egawa, T., Kawabata, K., Kawamoto, H., Amada, K., Okamoto, R., Fujii, N., Kishimoto, T., Katsura, Y., and Nagasawa, T. (2001). *The earliest stages of B cell development require a chemokine stromal cell-derived factor/pre-B cell growth-stimulating factor*. Immunity ***15***, 323-334. 10.1016/s1074-7613(01)00185-6.

117. Glodek, A.M., Honczarenko, M., Le, Y., Campbell, J.J., and Silberstein, L.E. (2003). *Sustained activation of cell adhesion is a differentially regulated process in B lymphopoiesis*. J Exp Med ***197***, 461-473. 10.1084/jem.20021477.

118. Tokoyoda, K., Egawa, T., Sugiyama, T., Choi, B.I., and Nagasawa, T. (2004). *Cellular niches controlling B lymphocyte behavior within bone marrow during development*. Immunity ***20***, 707-718. 10.1016/j.immuni.2004.05.001.

119. Griffiths, S.D., Goodhead, D.T., Marsden, S.J., Wright, E.G., Krajewski, S., Reed, J.C., Korsmeyer, S.J., and Greaves, M. (1994). *Interleukin 7-dependent B lymphocyte precursor cells are ultrasensitive to apoptosis*. J Exp Med ***179***, 1789-1797. 10.1084/jem.179.6.1789.

120. Cariappa, A., Mazo, I.B., Chase, C., Shi, H.N., Liu, H., Li, Q., Rose, H., Leung, H., Cherayil, B.J., Russell, P., von Andrian, U., and Pillai, S. (2005). *Perisinusoidal B cells in the bone marrow participate in T-independent responses to blood-borne microbes*. Immunity ***23***, 397-407. 10.1016/j.immuni.2005.09.004.

121. Sapoznikov, A., Pewzner-Jung, Y., Kalchenko, V., Krauthgamer, R., Shachar, I., and Jung, S. (2008). *Perivascular clusters of dendritic cells provide critical survival signals to B cells in bone marrow niches*. Nat Immunol ***9***, 388-395. 10.1038/ni1571.

122. Piao, W., Kasinath, V., Saxena, V., Lakhan, R., Iyyathurai, J., and Bromberg, J.S. (2021). *LTbetaR Signaling Controls Lymphatic Migration of Immune Cells*. Cells ***10***. 10.3390/cells10040747.

123. Stopfer, P., Obermeier, F., Dunger, N., Falk, W., Farkas, S., Janotta, M., Moller, A., Mannel, D.N., and Hehlgans, T. (2004). *Blocking lymphotoxin-beta receptor activation diminishes inflammation via reduced mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expression and leucocyte margination in chronic DSS-induced colitis*. Clin Exp Immunol ***136***, 21-29. 10.1111/j.1365-2249.2004.02402.x.

124. Browning, J.L., Allaire, N., Ngam-Ek, A., Notidis, E., Hunt, J., Perrin, S., and Fava, R.A. (2005). *Lymphotoxin-beta receptor signaling is required for the homeostatic control of HEV differentiation and function*. Immunity ***23***, 539-550. 10.1016/j.immuni.2005.10.002.

125. Madge, L.A., Kluger, M.S., Orange, J.S., and May, M.J. (2008). *Lymphotoxin-alpha 1 beta 2 and LIGHT induce classical and noncanonical NF-kappa B-dependent proinflammatory gene*

expression in vascular endothelial cells. J Immunol ***180***, 3467-3477. 10.4049/jimmunol.180.5.3467.

126. Pereira, J.P., An, J., Xu, Y., Huang, Y., and Cyster, J.G. (2009). *Cannabinoid receptor 2 mediates the retention of immature B cells in bone marrow sinusoids*. Nat Immunol ***10***, 403-411. 10.1038/ni.1710.

127. Gore, Y., Starlets, D., Maharshak, N., Becker-Herman, S., Kaneyuki, U., Leng, L., Bucala, R., and Shachar, I. (2008). *Macrophage migration inhibitory factor induces B cell survival by activation of a CD74-CD44 receptor complex*. J Biol Chem ***283***, 2784-2792. 10.1074/jbc.M703265200.

128. Klasen, C., Ziehm, T., Huber, M., Asare, Y., Kapurniotu, A., Shachar, I., Bernhagen, J., and El Bounkari, O. (2018). *LPS-mediated cell surface expression of CD74 promotes the proliferation of B cells in response to MIF*. Cell Signal ***46***, 32-42. 10.1016/j.cellsig.2018.02.010.

129. Bernhagen, J., Krohn, R., Lue, H., Gregory, J.L., Zernecke, A., Koenen, R.R., Dewor, M., Georgiev, I., Schober, A., Leng, L., Kooistra, T., Fingerle-Rowson, G., Ghezzi, P., Kleemann, R., McColl, S.R., Bucala, R., Hickey, M.J., and Weber, C. (2007). *MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment*. Nat Med ***13***, 587-596. 10.1038/nm1567.

130. Klasen, C., Ohl, K., Sternkopf, M., Shachar, I., Schmitz, C., Heussen, N., Hobeika, E., Levit-Zerdoun, E., Tenbrock, K., Reth, M., Bernhagen, J., and El Bounkari, O. (2014). *MIF promotes B cell chemotaxis through the receptors CXCR4 and CD74 and ZAP-70 signaling*. J Immunol ***192***, 5273-5284. 10.4049/jimmunol.1302209.

131. Matsumoto, M., Lo, S.F., Carruthers, C.J., Min, J., Mariathasan, S., Huang, G., Plas, D.R., Martin, S.M., Geha, R.S., Nahm, M.H., and Chaplin, D.D. (1996). *Affinity maturation without germinal centres in lymphotoxin-alpha-deficient mice*. Nature ***382***, 462-466. 10.1038/382462a0.

132. Bortnick, A., Chernova, I., Quinn, W.J., 3rd, Mugnier, M., Cancro, M.P., and Allman, D. (2012). *Long-lived bone marrow plasma cells are induced early in response to T cell-independent or T cell-dependent antigens*. J Immunol ***188***, 5389-5396. 10.4049/jimmunol.1102808.

133. Kang, H.S., Chin, R.K., Wang, Y., Yu, P., Wang, J., Newell, K.A., and Fu, Y.X. (2002). *Signaling via LTbetaR on the lamina propria stromal cells of the gut is required for IgA production*. Nat Immunol ***3***, 576-582. 10.1038/ni795.

134. Hu, S.H., Zhang, L.H., Gao, J., Guo, J.H., Xun, X.D., Xiang, X., Cheng, Q., Li, Z., and Zhu, J.Y. (2021). *NKG2D Enhances Double-Negative T Cell Regulation of B Cells*. Front Immunol ***12***, 650788. 10.3389/fimmu.2021.650788.

135. Askenase, M.H., Han, S.J., Byrd, A.L., Morais da Fonseca, D., Bouladoux, N., Wilhelm, C., Konkel, J.E., Hand, T.W., Lacerda-Queiroz, N., Su, X.Z., Trinchieri, G., Grainger, J.R., and Belkaid, Y. (2015). *Bone-Marrow-Resident NK Cells Prime Monocytes for Regulatory Function during Infection*. Immunity ***42***, 1130-1142. 10.1016/j.immuni.2015.05.011.

136. Wijdeven, R.H., van Luijn, M.M., Wierenga-Wolf, A.F., Akkermans, J.J., van den Elsen, P.J., Hintzen, R.Q., and Neefjes, J. (2018). *Chemical and genetic control of IFNgamma-induced MHCII expression*. EMBO Rep ***19***. 10.15252/embr.201745553.

137. Gurtner, A., Borrelli, C., Gonzalez-Perez, I., Bach, K., Acar, I.E., Nunez, N.G., Crepaz, D., Handler, K., Vu, V.P., Lafzi, A., Stirm, K., Raju, D., Gschwend, J., Basler, K., Schneider, C., Slack, E., Valenta, T., Becher, B., Krebs, P., Moor, A.E., and Arnold, I.C. (2023). *Active eosinophils regulate host defence and immune responses in colitis*. Nature ***615***, 151-157. 10.1038/s41586-022-05628-7.

138. de Bruin, A.M., Buitenhuis, M., van der Sluijs, K.F., van Gisbergen, K.P., Boon, L., and Nolte, M.A. (2010). *Eosinophil differentiation in the bone marrow is inhibited by T cell-derived IFN-gamma*. Blood ***116***, 2559-2569. 10.1182/blood-2009-12-261339.

139. Rais, M., Wild, J.S., Choudhury, B.K., Alam, R., Stafford, S., Dharajiya, N., and Sur, S. (2002). *Interleukin-12 inhibits eosinophil differentiation from bone marrow stem cells in an interferon-gamma-dependent manner in a mouse model of asthma*. Clin Exp Allergy ***32***, 627-632. 10.1046/j.0954-7894.2002.01350.x.

140. Sorg, U.R., Behnke, K., Degrandi, D., Reich, M., Keitel, V., Herebian, D., Deenen, R., Beyer, M., Schultze, J.L., Kohrer, K., Gabbert, H.E., Mayatepek, E., Haussinger, D., and Pfeffer, K. (2016). *Cooperative role of lymphotoxin beta receptor and tumor necrosis factor receptor p55 in murine liver regeneration*. J Hepatol ***64***, 1108-1117. 10.1016/j.jhep.2015.12.006.

141. De Trez, C., and Ware, C.F. (2008). *The TNF receptor and Ig superfamily members form an integrated signaling circuit controlling dendritic cell homeostasis*. Cytokine Growth Factor Rev ***19***, 277-284. 10.1016/j.cytogfr.2008.04.013.

142. Remouchamps, C., Boutaffala, L., Ganeff, C., and Dejardin, E. (2011). *Biology and signal transduction pathways of the Lymphotoxin-alphabeta/LTbetaR system*. Cytokine Growth Factor Rev ***22***, 301-310. 10.1016/j.cytogfr.2011.11.007.

143. Albarbar, B., Dunnill, C., and Georgopoulos, N.T. (2015). *Regulation of cell fate by lymphotoxin (LT) receptor signalling: Functional differences and similarities of the LT system to other TNF superfamily (TNFSF) members*. Cytokine Growth Factor Rev ***26***, 659-671. 10.1016/j.cytogfr.2015.05.001.

144. Merino, M.C., Montes, C.L., Acosta-Rodriguez, E.V., Bermejo, D.A., Amezcua-Vesely, M.C., and Gruppi, A. (2010). *Peritoneum from Trypanosoma cruzi-infected mice is a homing site of Syndecan-1 neg plasma cells which mainly provide non-parasite-specific antibodies*. Int Immunol ***22***, 399-410. 10.1093/intimm/dxq019.

145. Hoffmann, A., Kerr, S., Jellusova, J., Zhang, J., Weisel, F., Wellmann, U., Winkler, T.H., Kneitz, B., Crocker, P.R., and Nitschke, L. (2007). *Siglec-G is a B1 cell-inhibitory receptor that controls expansion and calcium signaling of the B1 cell population*. Nat Immunol ***8***, 695-704. 10.1038/ni1480.

146. Morris, D.L., and Rothstein, T.L. (1993). *Abnormal transcription factor induction through the surface immunoglobulin M receptor of B-1 lymphocytes*. J Exp Med ***177***, 857-861. 10.1084/jem.177.3.857.

147. Sen, G., Wu, H.J., Bikah, G., Venkataraman, C., Robertson, D.A., Snow, E.C., and Bondada, S. (2002). *Defective CD19-dependent signaling in B-1a and B-1b B lymphocyte subpopulations*. Mol Immunol ***39***, 57-68. 10.1016/s0161-5890(02)00047-0.

148. Bikah, G., Carey, J., Ciallella, J.R., Tarakhovsky, A., and Bondada, S. (1996). *CD5-mediated negative regulation of antigen receptor-induced growth signals in B-1 B cells*. Science ***274***, 1906-1909. 10.1126/science.274.5294.1906.

149. Ochi, H., and Watanabe, T. (2000). *Negative regulation of B cell receptor-mediated signaling in B-1 cells through CD5 and Ly49 co-receptors via Lyn kinase activity*. Int Immunol ***12***, 1417-1423. 10.1093/intimm/12.10.1417.

150. Khan, I.A., Hwang, S., and Moretto, M. (2019). *Toxoplasma gondii: CD8 T Cells Cry for CD4 Help*. Front Cell Infect Microbiol ***9***, 136. 10.3389/fcimb.2019.00136.

151. Brinkman, C.C., Iwami, D., Hritzo, M.K., Xiong, Y., Ahmad, S., Simon, T., Hippen, K.L., Blazar, B.R., and Bromberg, J.S. (2016). *Treg engage lymphotoxin beta receptor for afferent lymphatic transendothelial migration*. Nat Commun ***7***, 12021. 10.1038/ncomms12021.

152. Saxena, V., Piao, W., Li, L., Paluskievicz, C., Xiong, Y., Simon, T., Lakhan, R., Brinkman, C.C., Walden, S., Hippen, K.L., WillsonShirkey, M., Lee, Y.S., Wagner, C., Blazar, B.R., and Bromberg, J.S. (2022). *Treg tissue stability depends on lymphotoxin beta-receptor- and adenosine-*

receptor-driven lymphatic endothelial cell responses. Cell Rep ***39***, 110727. 10.1016/j.celrep.2022.110727.

153. Smith, T., Heger, A., and Sudbery, I. (2017). *UMI-tools: modeling sequencing errors in Unique Molecular Identifiers to improve quantification accuracy*. Genome Res ***27***, 491-499. 10.1101/gr.209601.116.

154. Martin, M. (2011). *Cutadapt removes adapter sequences from high-throughput sequencing reads*. EMBnet.journal ***17*** (*N 1*), 10-12. https://doi.org/10.14806/ej.17.1.200

155. Kopylova, E., Noe, L., and Touzet, H. (2012). *SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data*. Bioinformatics ***28***, 3211-3217. 10.1093/bioinformatics/bts611.

156. Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). *STAR: ultrafast universal RNA-seq aligner*. Bioinformatics ***29***, 15-21. 10.1093/bioinformatics/bts635.

157. Danecek, P., Bonfield, J.K., Liddle, J., Marshall, J., Ohan, V., Pollard, M.O., Whitwham, A., Keane, T., McCarthy, S.A., Davies, R.M., and Li, H. (2021). *Twelve years of SAMtools and BCFtools*. Gigascience ***10***. 10.1093/gigascience/giab008.

158. Liao, Y., Smyth, G.K., and Shi, W. (2014). *featureCounts: an efficient general purpose program for assigning sequence reads to genomic features*. Bioinformatics ***30***, 923-930. 10.1093/bioinformatics/btt656.

159. Love, M.I., Huber, W., and Anders, S. (2014). *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2*. Genome Biol ***15***, 550. 10.1186/s13059-014-0550-8.

160. Korotkevich, G., Sukhov, V., Budin, N., Shpak, B., Artyomov, M., and Sergushichev, A. (2016). *Fast gene set enrichment analysis*. bioRxiv. 10.1101/060012.

3.2 Antibody-mediated Protection in Lymphotoxin β Receptor-deficient Mice Against *Toxoplasma gondii*

Authors

Marcel Helle, Ursula R. Sorg, Anne Tersteegen, Daniel Degrandi, Klaus Pfeffer

Published in

Manuscript in preparation

Impact factor

-

Own contributions to this work

Overall: 90%

Conducted all of the following experiments:

T. gondii infection experiments, serum transfer experiments, generation and quantification of immune and control sera, flow cytometry, qRT-PCRs (parasite load), cytokine analysis, detection of *T. gondii* specific antibodies

Other major contributions:

Design of experiments, manuscript preparation, data analysis

Antibody-mediated Protection in Lymphotoxin β Receptor-deficient Mice Against *Toxoplasma gondii*

Marcel Helle[1], Ursula R. Sorg[1], Anne Tersteegen[2], Daniel Degrandi[1], Klaus Pfeffer[1]*

[1]Institute of Medical Microbiology and Hospital Hygiene, Heinrich Heine University, Düsseldorf, Germany.

[2]Institute for Biochemistry and Cell Biology, Otto-von-Guericke-University, Magdeburg, Germany

*: To whom correspondence should be addressed

Prof. Dr. Klaus Pfeffer
Institute of Medical Microbiology and Hospital Hygiene
Heinrich Heine University Düsseldorf
Universitätsstraße 1
40225 Düsseldorf, Germany
Phone: +49 (0) 211 81-12459
Fax: +49 (0) 211 81-15906
Email: klaus.pfeffer@hhu.de

Keywords: lymphotoxin beta receptor, *Toxoplasma gondii*, immunity, adaptive immune response, B cells, antibody, infection, mouse

Abstract

The lymphotoxin beta receptor, a member of the TNF-receptor superfamily, is vital for lymphoid organ development, immune cell homeostasis, and structured immune responses. LTβR deficient mice show significant immune deficiencies, including the absence of lymph nodes, an altered splenic architecture and differences in immune cell populations, leading to increased susceptibility to various pathogens.

This includes *Toxoplasma gondii*, an obligate intracellular protozoan parasite. *T. gondii* has infected approximately 30 % of the global human population and poses severe risks to immunocompromised individuals and fetuses. While IL-12-driven IFNγ production is critical for controlling acute infection, our previous research showed that LTβR-deficient mice exhibit impaired cell-autonomous and adaptive immune responses against *T. gondii*.

In this study, we explored antibody-mediated protection in LTβR$^{-/-}$ mice through passive immunization experiments. Although the immune serum transfer significantly prolonged survival of LTβR$^{-/-}$ mice, it did not prevent mortality or reduce parasite burden, likely due to the intracellular nature of *T. gondii*. Interestingly, in WT mice, immune serum transfer led to a reduction in specific *T. gondii* IgM and IgG antibodies, particularly against GRA7 and GRA8 antigens, possibly due to IgG-mediated suppression through mechanisms such as epitope masking or trogocytosis. Anti-ROP1c-specific antibodies were less affected, likely due to lower anti-ROP1c IgG levels in the transferred serum. These findings suggest that while immune serum transfer can offer partial protection in LTβR-deficient mice, it may also selectively suppress specific antibody responses, highlighting the need to carefully consider the dynamics of antibody transfer in experimental and therapeutic strategies.

Additionally, we investigated the role of LTβR signaling in specific immune cells during *T. gondii* infection, using mouse models with targeted LTβR inactivation in DCs (LTβR$^{fl/fl}$ x CD11c-Cre, from here on referred to as LTβR-CD11cCre) and myeloid cells (LTβR$^{fl/fl}$ x LysM-Cre, from here on referred to as LTβR-LysMCre). Preliminary results show that LTβR deletion in myeloid cells was associated with a non-significant trend toward reduced survival, whereas deletion in DCs did not increase susceptibility compared to WT mice. Female WT and LTβR-LysMCre mice had comparable survival rates, while male LTβR-LysMCre mice tended to exhibit shorter survival during the acute stage and WT males tended to display prolonged survival during the chronic stage of *T. gondii* infection, indicating potential genotype- and sex-specific effects. These findings suggest that LTβR mediated signaling in myeloid cells may contribute to survival during acute infection, although further research is needed to clarify the significance of these observations.

Introduction

The lymphotoxin beta receptor (LTβR) is part of the tumor necrosis factor (TNF)-receptor superfamily and expressed by various cell types, including epithelial, endothelial, stromal, and myeloid cells such as monocytes and macrophages [1,2], neutrophils [3], dendritic cells (DCs) [4] and mast cells, but is absent from lymphoid cells [5]. In contrast, the two known ligands of the LTβR are found on lymphoid cells, indicating that LTβR signaling predominantly occurs in a paracrine or juxtracrine manner. The $LT\alpha_1\beta_2$ ligand specifically binds only to the LTβR, whereas LIGHT (homologous to **l**ymphotoxins, exhibits **i**nducible expression, and competes with HSV **g**lycoprotein D for **h**erpes virus entry mediator, a receptor expressed by **T** lymphocytes) also interacts with the herpes virus entry mediator (HVEM) receptor and soluble decoy receptor 3 (DcR3), thus creating a larger signaling network [5-8]. LTβR signaling primarily activates the canonical and alternative nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) pathways [9] but can also involve extracellular signal-regulated kinase (Erk) and c-Jun N-terminal kinase (JNK) signaling pathways [10].

The LTβR is crucial for lymphoid organ development during embryogenesis and for maintaining lymphoid organ structure and immune cell homeostasis in adulthood [11]. LTβR-deficient mice ($LT\beta R^{-/-}$) lack lymph nodes and Peyer's patches (PPs) [11,12], have fewer DCs [4,13], and exhibit a significant reduction in natural killer (NK) and NKT cell numbers [11,14-17]. LTβR signaling also affects the thymus by regulating thymocyte migration and selection [18], and the spleen by maintaining B and T cell compartmentalization, germinal center formation, and the homeostasis of follicular DCs and macrophage subpopulations [11,12,19-24]. The high susceptibility of $LT\beta R^{-/-}$ mice to various pathogens due to defective immune responses highlights its essential role for a functional host defense [11,17,25-30]. Furthermore, $LT\beta R^{-/-}$ mice show signs of autoimmunity, such as splenomegaly, increased perivascular lymphocytic infiltrations in non-lymphoid organs, autoantibody production, and heightened baseline immune activation [11,12,17,26,31,32]. Thus, the LTβR is receiving great interest regarding its role, but also as a potential therapeutic target, in autoimmune diseases [3,33-35] and cancer [36,37].

Toxoplasma gondii is a highly successful obligate intracellular protozoan parasite that has infected an estimated 30% of the global human population, with no human vaccine currently available [38,39]. Although members of the mammalian *Felidae* family serve as the definitive hosts for this eukaryotic parasite, *T. gondii* can infect virtually all warm-blooded vertebrates and undergo asexual reproduction in these intermediate hosts, including humans [40]. While toxoplasmosis, the disease caused by *T. gondii* infection, typically results in mild, flu-like symptoms in immunocompetent individuals, it can be devastating for immunocompromised patients. First-time infection during pregnancy can lead to

abortion of the fetus or can cause severe health issues in the child, such as encephalitis, myocarditis, and pneumonia with potentially long-term effects. [41].

During acute infection, *T. gondii* exists as so-called 'tachyzoites', which can rapidly replicate and disseminate within the host. Upon invading a host cell, the parasite becomes engulfed in a parasitophorous vacuole (PV), which is derived from the host cell membrane but modified with *T. gondii*-derived molecules. This creates a replicative niche within the cell where the parasite is protected from the host immune response and supplied with nutrients [42]. Typically, within two weeks of infection in mice and humans, the immune system recognizes and begins to control the infection. At this stage, tachyzoites differentiate into slower-replicating 'bradyzoites'. As bradyzoites, encapsulated in cysts, preferentially within brain and muscle tissue, and by maintaining a low profile to evade decisive immune responses, *T. gondii* can persist lifelong in the host as chronic infection with the risk of reactivation and retransition into acute toxoplasmosis [43].

A coordinated and efficient response involving innate, adaptive, and cell autonomous immunity is crucial for controlling *T. gondii* infection [44-47]. *T. gondii*-derived molecules are detected by Toll-like receptors (TLRs) [48-51] and the C-C chemokine receptor type 5 (CCR5) receptor [52,53] on DCs and macrophages, leading to the production of proinflammatory cytokines, including interleukin 12 (IL-12). An early and robust expression of interferon-gamma (IFNγ) by NK cells [54,55] and innate lymphoid cells 1 (ILC1) [56], driven by IL-12, is vital for the induction of potent cell autonomous immune mechanisms, such as the production of murine guanylate-binding proteins (mGBPs), which directly target and destroy the PV and the parasite within [57-61]. IL-12 also drives the characteristic T helper 1 (Th-1) response against intracellular pathogens, resulting in additional IFNγ production by CD4 (and CD8) T cells [54,62,63]. Neutrophils have also been identified as sources of IFNγ against *T. gondii*, independent of IL-12 [64].

While the role of B cell- and antibody-mediated protection has been relatively neglected compared to T cell- and cell-autonomous immunity, it nevertheless contributes significantly to the host defense against intracellular pathogens, including *T. gondii* [65-67]. Antibodies provide protection by opsonizing *T. gondii* parasites and blocking host cell invasion [68-73]. The antibody response is dependent on CD4 T cells, highlighting the necessity of a coordinated immune response against this pathogen [74,75]. Additionally, natural antibodies with germline-like characteristics, which can be secreted spontaneously without exogenous antigenic stimulation, are part of the "first line of defense" against invading pathogens as they are present before infection [76]. While natural antibodies have multiple functions and provide protection against various pathogens, natural IgM was found to be non-protective against *T. gondii* invasion [68].

In general, protozoan parasites elicit a certain degree of immunosuppression in their hosts through different mechanisms, for example by inducing apoptosis in specific B cell subpopulations [77,78], which serves their survival and reproduction [79]. Reported mechanisms that partially suppress antibody responses also include the induction of suppressor macrophages and/or T cells (depending on the invading parasitic species [80]), the release of soluble factors from *T. gondii*-infected monocytes [81], and the temporary disruption of the splenic architecture [74]. *T. gondii* also targets essential IFNγ-mediated mechanisms directly by secreting the parasite-derived molecule TgIST, which translocases to the host cell nucleus and blocks IFNγ-dependent [82] (and type I IFN-dependent [83]) gene expression.

Overall, the interplay between the humoral immune response and protozoan parasites is diverse and not fully understood [79]. While the humoral immune response may contribute to host protection, the invading parasites strive to overcome this protection which is also reflected in the difficulties in vaccine development [39]. In addition to active immunization, which aims to stimulate an endogenous antibody response, passive immunization aims to provide immediate protection by transferring pre-formed antibodies from an immune donor to a recipient. In the literature, both approaches have led to ambiguous results, as some studies confirmed a protective effect [67,70,75,84-87] while others report no enhancement of the humoral response in immunized subjects [88-90].

In our previous studies, we investigated the susceptibility of LTβR$^{-/-}$ mice to *T. gondii* infection and found that cell-autonomous immunity, particularly the expression of IFNγ and mGBPs, as well as adaptive immunity, were severely impaired in the absence of LTβR signaling [17,26]. LTβR deficiency led to absent *T. gondii*-specific IgG and reduced IgM antibodies in the serum, indicating defective affinity maturation and immunoglobulin class-switching in these mice [17].

In this study, we extended our investigation of the role of LTβR signaling in the humoral immune response during protozoan parasite infection by conducting passive immunization experiments. While passive immunization conferred some protective effects, it did not significantly limit parasite replication in either WT or LTβR$^{-/-}$ mice. Interestingly, recipients of WT immune serum exhibited reduced levels of *T. gondii*-specific IgM and IgG antibodies in their serum. This specific reduction may be attributed to the potential immunosuppressive effect of transferred high-affinity IgG antibodies, which is a critical factor to consider for future experiments and therapeutic strategies. Furthermore, while cytokine expression profiles and splenic immune cell populations in LTβR$^{-/-}$ mice displayed the known alterations, these parameters were not significantly affected by the immune serum transfer. These findings highlight the complex relationship between humoral immunity and intracellular parasites, underscoring the challenges of enhancing protection against *T. gondii*.

Additionally, we investigated the requirements of LTβR signaling in DCs (LTβR-CD11cCre) and myeloid cells (LTβR-LysMCre) for *T. gondii* defense to further elucidate how LTβR signaling modulates the immunes response against this parasite. Preliminary results show a trend towards reduced survival in LTβR-LysMCre mice, indicating a potential role of LTβR signaling in myeloid cells during infection. Moreover, potential sex-specific effects were observed: female WT and LTβR-LysMCre mice had similar survival rates, whereas male LTβR-LysMCre exhibited a trend of increased susceptibility during the acute stage of *T. gondii* infection. In contrast, male WT mice exhibited a trend of increased survival compared to females and LTβR-LysMCre mice during the chronic stage of infection. While preliminary, these results warrant further investigation to confirm the observed trends.

Results

A passive immunization experiment was performed to investigate antibody-mediated protection in LTβR$^{-/-}$ mice during *T. gondii* infection. As depicted in Fig. 1a, the required immune serum was generated from *T. gondii*-infected WT donor mice 14 days postinfection (p.i.) and was tested for *T. gondii*-specific IgM and IgG antibodies before usage, whereas control serum was generated from uninfected WT littermates. Recipient WT and LTβR$^{-/-}$ mice received immune or control serum *via* intraperitoneal (i.p.) injections before (day -1) and on day 2, 5 and 8 p.i., and were sacrificed for analysis one day after each serum transfer (day 3, 6 and 9 p.i.). Uninfected WT and LTβR$^{-/-}$ mice that did not receive serum served as additional controls.

Passive immunization provides a protective effect but does not prevent mortality and does not significantly reduce parasite burdens in LTβR$^{-/-}$ mice

LTβR$^{-/-}$ mice are highly susceptible to intraperitoneal *T. gondii*-infection and do not survive beyond day 14 p.i., as shown in our previous studies [17,26]. The transfer of immune serum significantly, albeit briefly, prolonged the survival of LTβR$^{-/-}$ recipient mice compared to those receiving control serum (Fig. 1b). Nevertheless, all animals of both LTβR$^{-/-}$ cohorts died before reaching day 14 p.i. (immune serum) and day 12 p.i. (control serum) while most animals of both WT cohorts survived the *T. gondii* infection until the end of the experiment on day 21 p.i. While not statistically significant, the immune serum also had a protective effect on the survival of WT recipient mice compared to those receiving control serum. IFNγR$^{-/-}$ mice are even more susceptible to *T. gondii* infection than LTβR$^{-/-}$ mice and served as infection controls.

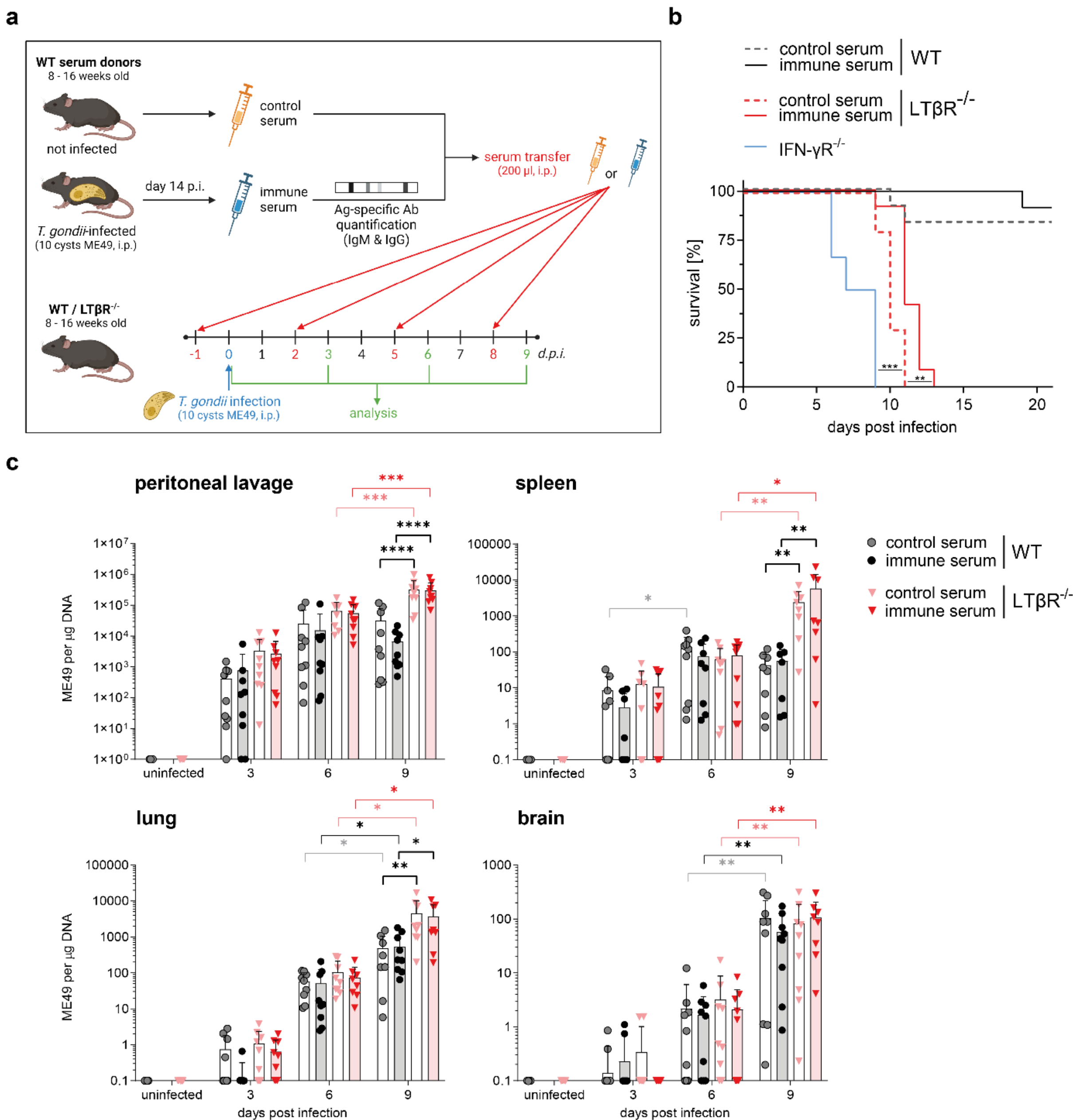


Figure 1: Passive immunization offers partial protection but fails to prevent mortality nor significantly reduces parasite burdens in LTβR$^{-/-}$ mice. (a) Schematic representation of the passive immunization model. Control serum was derived from uninfected WT mice, while immune serum was obtained on d14 p.i. from WT mice infected with *T. gondii*. *T. gondii*-specific IgM and IgG antibodies were semi-quantified using antigen-loaded line blots. WT and LTβR$^{-/-}$ mice received multiple i.p. injections of either immune or control serum on the days indicated. Mice were infected i.p. with bradyzoites from 10 lysed cysts of the *T. gondii* ME49 strain and were sacrificed at designated time points for analysis. **(b)** Survival of *T. gondii*-infected WT and LTβR$^{-/-}$ mice that received immune or control serum (n = 12 for all groups except LTβR$^{-/-}$ control serum: n = 10 and IFN-γR$^{-/-}$: n = 6). Statistical analysis was conducted using the log-rank (Mantel-Cox) test. **(c)** Parasite burden was assessed *via* *T. gondii*-specific qPCR targeting the *TgB1*-gene in the spleen, lung, brain, and peritoneal lavage samples (n ≥ 8 for all tissue and cohorts). Uninfected WT and LTβR$^{-/-}$ mice served as controls. All data shown represent at least three independent experiments; symbols represent individual animals and columns represent means ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

The parasite burden in the peritoneal lavage, spleen and lung were significantly increased in LTβR$^{-/-}$ mice on day 9 p.i. compared to WT mice, and there was no significant difference between immune serum and control serum recipients in both genotypes (Fig. 1c). The peritoneal lavage had both the highest number of parasites compared to other investigated tissue and the most significant difference between LTβR$^{-/-}$ and WT animals on day 9 p.i. Conversely, the brain exhibited the overall lowest parasite burden in both WT and LTβR$^{-/-}$ mice, with no significant difference between the two genotypes. Additionally, parasite numbers in the peritoneal lavage and spleen of WT mice did not increase further from day 6 to day 9 post-infection, in contrast to lung and brain and all investigated tissues from LTβR$^{-/-}$ mice.

Immunized WT mice have reduced amounts of specific *T. gondii*-specific IgM and IgG serum antibodies compared to control serum recipients

Serum from WT donor mice (day 14 p.i.) was analyzed for *T. gondii*-specific IgM and IgG *via* commercially available immunoblots (“line blots”, *Recom*line, Mikrogen Diagnostics) loaded with eight selected recombinant *T. gondii* tachyzoite and bradyzoite antigen proteins (ROP1c, MIC3, GRA7, GRA8, p30, MAG1, GRA1 and rSAG1). In our serum transfer model, antibodies against four of these eight *T. gondii*-antigens were detectable: anti-ROP1c, anti-GRA7, anti-GRA8 and anti-MAG1. As quality control, all generated immune serum batches were analyzed *via* serial dilution for *T. gondii*-specific IgM and IgG before use (Fig. 2a, Suppl. Fig. 1). Overall, the immune serum contained higher levels of *T. gondii*-specific IgM than IgG, with less anti-MAG1 IgM compared to anti-ROP1c, anti-GRA7 and anti-GRA8 IgM, and less anti-ROP1c IgG compared to anti-GRA7, anti-GRA8 and anti-MAG1 IgG (Fig 2a, Suppl. Fig. 1).

We previously described reduced amounts of *T. gondii*-specific IgM and the absence of *T. gondii*-specific IgG antibodies from the serum of infected LTβR$^{-/-}$ mice [17]. In line with these observations, here also, no *T. gondii*-specific antibodies were detectable at all in the serum from LTβR$^{-/-}$ control serum recipients on day 9 p.i. (Fig. 2b). In contrast, LTβR$^{-/-}$ immune serum recipients showed low amounts of IgM directed against ROP1c, GRA7, GRA8, and MAG1 and only very low amounts of anti-GRA7 IgG compared to WT mice. In both WT cohorts (immune and control serum recipients) IgM and IgG against four antigens were detectable, with the exceptions of anti-ROP1c IgG in WT immune serum recipients and anti-MAG1 IgG in WT control serum recipients, which were not detectable. In addition to their unique occasional display of anti-ROP1c IgG, surprisingly, WT control serum recipients had equal or significantly higher amounts of parasite-specific IgM and IgG compared to WT immune serum recipients. This contrasts with the LTβR$^{-/-}$ cohorts, where control serum recipients consistently had lower amounts of parasite-specific IgM compared to the immune serum recipients.

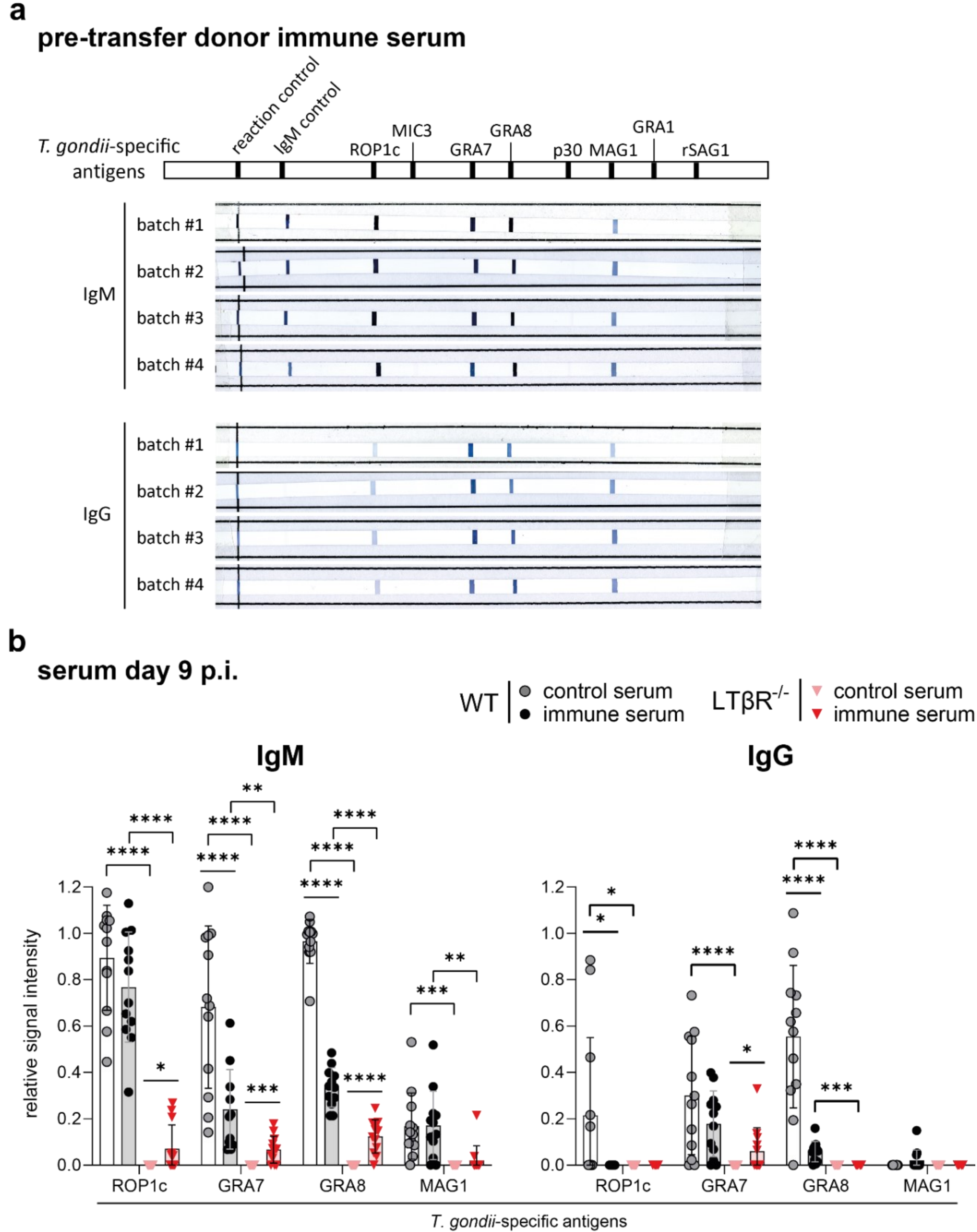


Figure 2: *T. gondii*-specific IgM and IgG serum antibodies are absent in LTβR$^{-/-}$ control serum recipients and reduced in WT immune serum recipients on day 9 p.i. *T. gondii*-specific serum IgM and IgG was quantified using commercially available line blots loaded with recombinant *T. gondii* antigens (Recomline, Mikrogen, Germany). **(a)** Line blot analysis of pre-transfer WT donor immune serum batches of day 14 p.i., final dilution 1:100. IgG control bands are of unknown specificity and did not react with murine samples, as the assay was originally designed for human samples. **(b)** Line blot analysis of serum from WT and LTβR$^{-/-}$ immune and control serum recipients on day 9 p.i. Band signal intensities were determined using FIJI/ImageJ software and normalized to pre-transfer WT donor immune serum levels (n = 12 mice). All data shown are summarized from four independent experiments; symbols represent individual animals and columns represent means ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

To verify the presence of transferred antibodies in the serum of recipient mice, immune serum was administered *via* a single i.p. injection to two uninfected WT mice. After 24 hours, the serum was collected and analyzed. While the overall antibody transfer *via* i.p. injection was successful, antibody levels were lower compared to the pre-transfer immune serum (Suppl. Fig. 2). Anti-ROP1c, anti-GRA7, and anti-GRA8 IgM were clearly detectable, while anti-MAG1 IgM was only weakly detectable. Among the IgGs, only anti-GRA7 was clearly present, anti-GRA8 was weakly detectable, and anti-ROP1c and anti-MAG1 were not detectable.

Additionally, pre-transfer control and immune sera were analyzed for proinflammatory cytokines. While slight variations were observed across the four batches of generated serum, most cytokine concentrations were similar between the control and immune sera (Suppl. Fig. 3). Notable exceptions were elevated levels of IFNγ, TNFα (except in batch #3), and IL-6 in the immune sera.

Passive immunization does not alter proinflammatory cytokine concentrations in the serum of WT and LTβR$^{-/-}$ cohorts during *T. gondii* infection

Proinflammatory cytokine levels in the serum of uninfected LTβR$^{-/-}$ mice were elevated compared to WT mice (Fig. 3), consistent with our previous findings [17]. The most pronounced differences were observed for IFNγ. While IFNγ concentrations were comparable between genotypes on day 3 p.i., they were significantly higher in WT mice on day 6 p.i. compared to LTβR$^{-/-}$ mice, which maintained basal IFNγ levels until day 9 p.i. A similar, though less pronounced, pattern was observed for TNFα. By day 9 p.i., IL-12p70 and particularly MCP-1 (CCL-2) concentrations were significantly higher in LTβR$^{-/-}$ cohorts compared to WT cohorts. Notably, immune serum recipients did not exhibit significant changes in cytokine patterns compared to control serum recipients. However, LTβR$^{-/-}$ immune serum recipients showed a trend towards reduced levels of IL-1α, IL-12p70, GM-CSF, and MCP-1 on day 9 p.i. compared to control serum recipients.

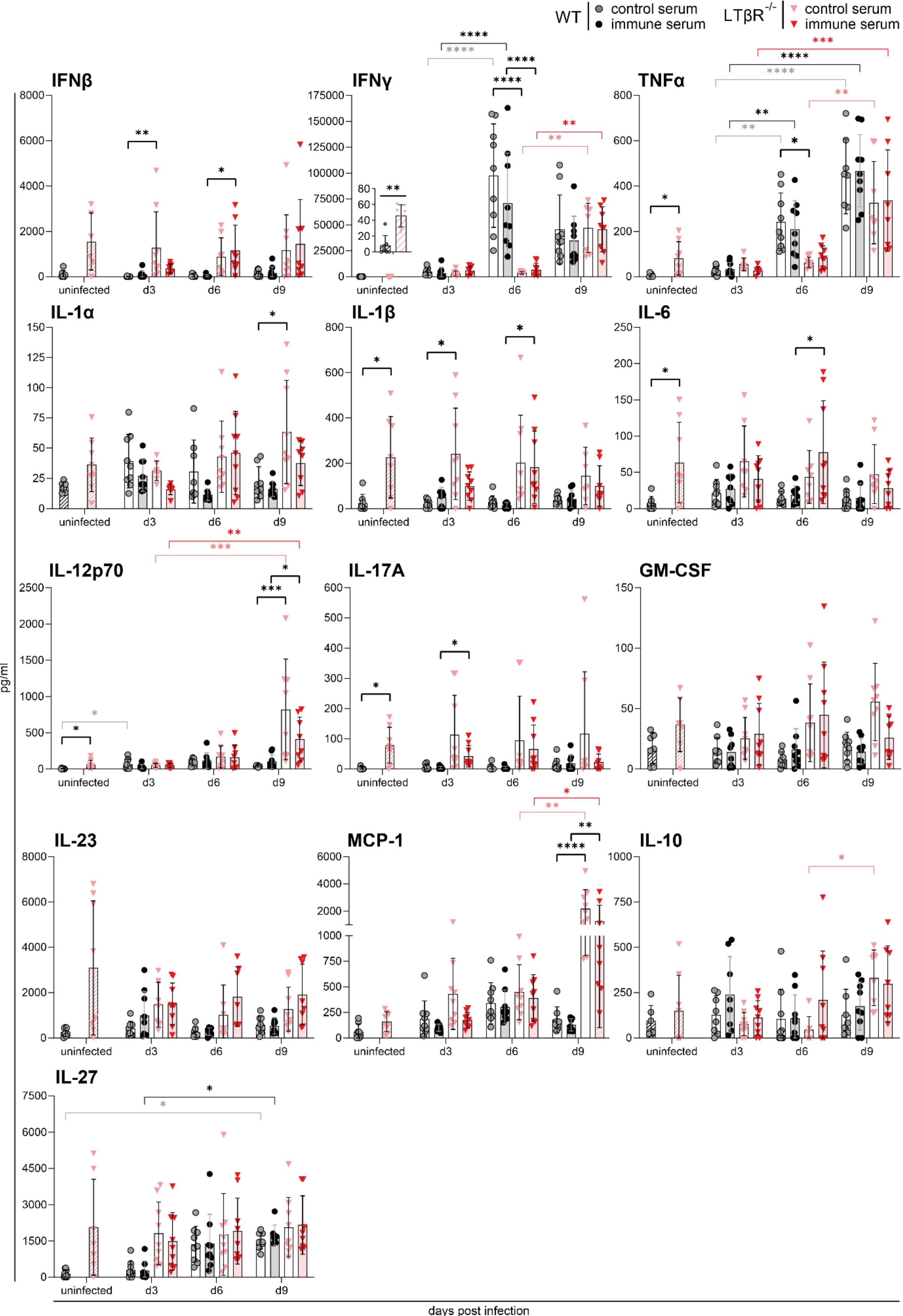


Figure 3: Passive immunization does not alter proinflammatory cytokine levels in the serum of WT and LTβR-/- cohorts during *T. gondii* infection. Cytokines measured in the serum of uninfected and infected WT (n ≥ 9) and LTβR-/- (n ≥ 9) mice *via* bead-based multiplex assay (LegendPlex, BioLegend, USA). All data shown represent four

independent experiments; symbols represent individual animals and columns represent means ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Passive immunization does not alter the splenic immune cell composition of WT and LTβR$^{-/-}$ cohorts during *T. gondii* infection

No significant differences were observed in splenic immune cell frequencies between immune and control serum recipients within both WT and LTβR$^{-/-}$ cohorts, with the exception of pan-T cells (CD3^{+}) which were elevated in LTβR$^{-/-}$ immune serum recipients on day 9 p.i. (Fig. 4). Contrary to previous findings, there was no significant reduction in B cell frequencies on day 9 p.i., whereas earlier studies showed a clear decrease in splenic B cell numbers in both WT and LTβR$^{-/-}$ mice by day 10 p.i. [17]. During infection, pan-T cell frequencies decreased in WT cohorts but remained significantly elevated in LTβR$^{-/-}$ cohorts on day 9 p.i. The ratio of CD4 to CD8 T cell subsets also shifted, with all cohorts showing an increase in CD4 and a decrease in CD8 frequencies; the latter was significantly lower in LTβR$^{-/-}$ cohorts compared to WT cohorts on day 9 p.i. Activated (CD25^{+}) T cells were significantly reduced in uninfected LTβR$^{-/-}$ mice, and although both genotypes exhibited increased activated T cell frequencies during infection, this increase occurred earlier in WT cohorts (from day 3 to day 6 p.i.) compared to LTβR$^{-/-}$ cohorts (from day 6 to day 9 p.i.).

Furthermore, the spleens of uninfected LTβR$^{-/-}$ mice exhibited the expected virtual absence of NK [16] and NKT cells [14,17], and reduced DC populations [4,12,91] compared to WT animals. This was observed for pan-DCs as well as the plasmacytoid DC subpopulation (pDCs, Fig. 4). Notably, the differences between WT and LTβR$^{-/-}$ animals in NK cell, NKT cell, pan-DC and pDC frequencies became less distinct during *T. gondii* infection, as frequencies declined and variability increased in the WT cohorts. Neutrophil frequencies remained stable until day 9 p.i., on which they increased across all cohorts but were significantly higher in WT compared to LTβR$^{-/-}$ animals.

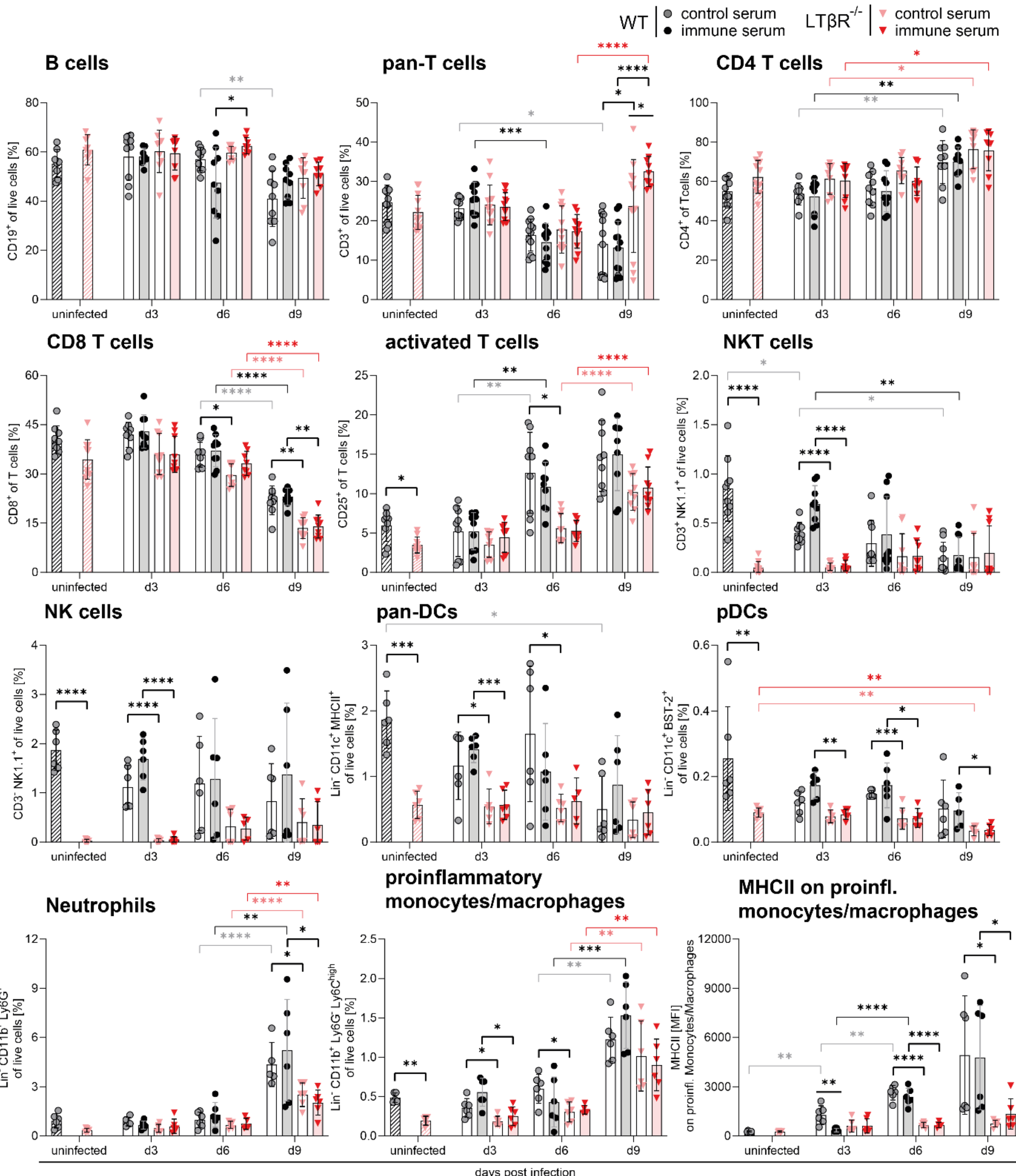


Figure 4: Analysis of splenic immune cell populations after passive immunization and *T. gondii* infection. Based on surface marker staining and flow cytometry (gating strategy: Suppl. Fig. 4 & 5), the following immune cell populations were identified in the spleen of WT (n ≥ 6) and LTβR$^{-/-}$ (n ≥ 6) serum recipients: B cells (CD19$^+$ CD3$^-$), pan-T cells (CD19$^-$ CD3$^+$), CD4 T cells (CD19$^-$ CD3$^+$ CD4$^+$ CD8$^-$, % of pan-T cells), CD8 T cells (CD19$^-$ CD3$^+$ CD4$^-$ CD8$^+$, % of pan-T cells), activated T cells (CD19$^-$ CD3$^+$ CD25$^+$, % of pan-T cells), NKT cells (CD3$^+$ NK1.1$^+$), NK cells (CD19$^-$ CD3$^-$ CD11b$^+$ CD11c$^+$ NK1.1$^+$), pan-DCs (CD19$^-$ CD3$^-$ NK1.1$^-$ CD11c$^+$ MHCII$^+$), pDCs (CD19$^-$ CD3$^-$ CD11c$^+$ B220$^+$ BST2$^+$), neutrophils (CD19$^-$ CD3$^-$ NK1.1$^-$ CD11b$^+$ Ly6G$^+$), and proinflammation monocytes/macrophages (CD19$^-$ CD3$^-$ NK1.1$^-$ CD11b$^+$ CD11c^{low} Ly6G$^-$ Ly6C^{high}). Additionally, the MHCII median fluorescence intensity (MFI) was determined on proinflammatory monocytes/macrophages. Unless stated otherwise, frequencies are reported as live cell frequencies. All data shown represent at least two independent experiments; symbols represent individual animals and columns represent means ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

Moreover, the frequencies of splenic proinflammatory (Ly6C^{high}) monocytes/macrophages were significantly reduced in uninfected LTβR$^{-/-}$ mice compared to WT mice. This initial difference diminished as the infection progressed, with all cohorts showing an increase from day 6 to day 9 p.i. However, MHCII expression on these proinflammatory monocytes/macrophages was consistently higher in WT mice, particularly on day 6 p.i., whereas LTβR$^{-/-}$ cohorts did not exhibit a significant increase in MHCII expression throughout the infection. Notably, WT control serum recipients showed an early rise in MHCII expression on day 3 p.i., a response absent in WT immune serum recipients.

Additionally, the immune serum did not affect spleen weight in either WT or LTβR$^{-/-}$ mice. Although spleen weight increased during *T. gondii* infection across all cohorts, WT mice had significantly higher spleen weights on day 9 p.i. compared to LTβR$^{-/-}$ mice, consistent with our previous findings (Suppl. Fig. 6, [17]).

LTβR inactivation in myeloid cells shows a trend towards reduced survival in male mice during acute infection

In addition to investigating complete LTβR-deficient mice (LTβR$^{-/-}$), we examined the impact of LTβR-deficiency specifically in DCs (LTβR-CD11cCre) and myeloid cells (LTβR-LysMCre) on mouse survival during *T. gondii* infection. Mice with ubiquitous LTβR inactivation (LTβR$^{fl/fl}$ x Cre-deleter, from here on referred to as LTβR-CreDeleter) served as positive controls for the Cre/loxP-mediated LTβR inactivation, while LTβR$^{fl/fl}$ mice were used as negative controls.

The acute stage of infection of a ME49 *T. gondii* infection lasts approximately 14 days and is characterized by rapid tachyzoite proliferation and severe infection symptoms in mice, including weight loss and behavioral changes such as reduced activity and fur grooming. As the host immune pressure intensifies, tachyzoites begin transitioning into bradyzoites, initiating the chronic stage of infection. During this phase, mice gradually regain weight and exhibit improved activity compared to the acute stage of infection, usually to the point of complete recovery.

During acute *T. gondii* infection, LTβR-LysMCre mice showed a trend towards intermediate survival, situated between the higher susceptibility of LTβR$^{-/-}$ and LTβR-CreDeleter strains, and the lower susceptibility of WT and LTβR$^{fl/fl}$ control strains (Fig. 5a). Notably, LTβR-LysMCre, LTβR-CD11cCre, LTβR$^{fl/fl}$, and WT mice exhibited significantly prolonged survival compared to LTβR$^{-/-}$ and LTβR-CreDeleter mice, which succumbed entirely by day 11 p.i. and did not reach the chronic stage of infection. Except for one WT mouse, all WT, LTβR$^{fl/fl}$, and LTβR-CD11cCre mice survived the acute stage, whereas several LTβR-LysMCre mice succumbed during this period.

During the chronic stage of *T. gondii* infection, WT and LTβR$^{fl/fl}$ mice began to succumb, with approximately 30 % surviving by the end of the observation period. LTβR-LysMCre mice continued to exhibit a trend of increased susceptibility, whereas LTβR-CD11cCre mice showed a trend of prolonged survival compared to WT mice during chronic infection. Additionally, the survival curves of LTβR-LysMCre and LTβR-CD11cCre mice differed significantly.

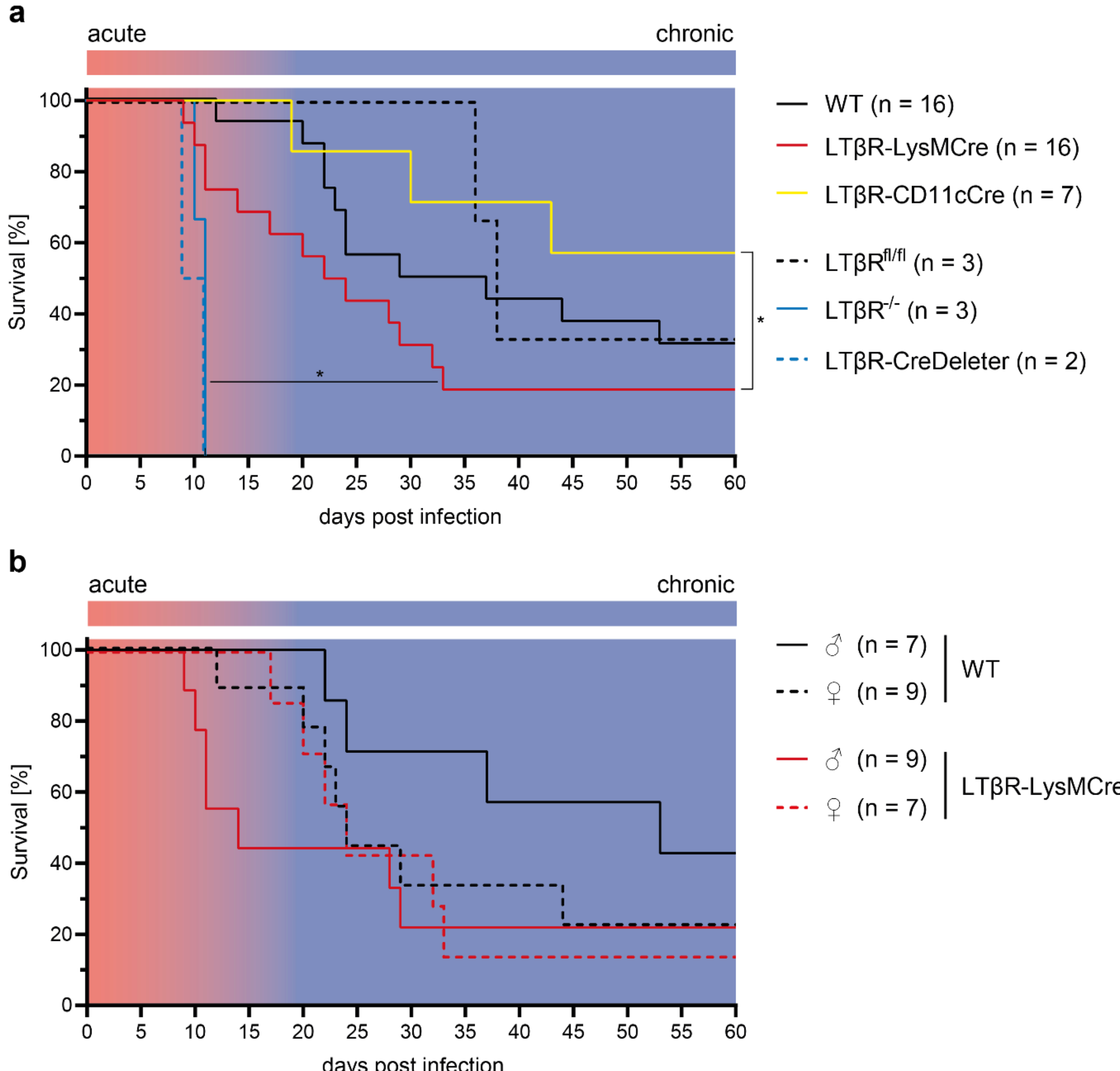


Figure 5: LTβR deletion in myeloid cells shows a trend towards reduced survival in LTβR-LysMCre mice. (a) Survival of *T. gondii*-infected (ME49, bradyzoites from 10 cysts, i.p.) WT (n = 16), LTβR-LysMCre (n = 16), LTβR-CD11cCre (n = 7), LTβR$^{fl/fl}$ (n = 3), LTβR$^{-/-}$ (n = 3), and LTβR-CreDeleter (n = 3) mice. **(b)** Survival of WT, LTβR-LysMCre and LTβR-CD11cCre mice according to sex. Statistical analysis was conducted using the log-rank (Mantel-Cox) test. *, $P < 0.05$

When analyzed by sex, male LTβR-LysMCre mice showed a tendency towards increased mortality during the acute stage of infection, while females appeared less susceptible and more similar to the survival curves of WT mice (Fig. 5b). Female LTβR-LysMCre as well as female WT mice succumbed during the early chronic stage, between days 14 and 30 p.i. Male WT mice also began to succumb during chronic infection, albeit at a lower rate compared to the females. Due to limited mouse

availability, only female LTβR-CD11cCre mice were studied so far, and their survival curve appeared similar to that of male WT mice (data not shown).

All WT, LTβR-LysMCre and LTβR-CD11cCre mice experienced severe weight loss during *T. gondii* infection, which temporarily stabilized around day 14 p.i. but remained below initial levels for the rest of the experiment (Suppl. Fig. 7). LTβR$^{-/-}$, LTβR$^{fl/fl}$, and LTβR-CreDeleter mice displayed similar patterns of weight loss (data not shown).

Discussion

Since LTβR$^{-/-}$ mice display profound defects in their endogenous production of *T. gondii*-specific IgM and IgG antibodies [17], we aimed to investigate the effect of reconstituted antibody protection in LTβR$^{-/-}$ mice *via* a passive immunization model. Although the transfer of immune serum significantly prolonged survival in LTβR$^{-/-}$ recipient mice, it did not prevent their eventual death. A similar, but statistically non-significant trend of prolonged survival was also observed for WT immune serum recipients. However, parasite burdens in the spleen, lung, peritoneal lavage, and brain were not significantly reduced in LTβR$^{-/-}$ or WT immune serum recipient mice. This limited effect of passive immunization is likely due to the obligate intracellular nature of *T. gondii*, which prevents its direct targeting by antibodies. While there are short periods in the life cycle of *T. gondii* where it is exposed to the extracellular space and humoral immune responses, IFNγ-driven cell-autonomous immunity likely provides a more potent defense against this parasite [46,47,92]. Despite slightly elevated basal levels of several proinflammatory cytokines under steady-state conditions, LTβR$^{-/-}$ mice are unable to efficiently upregulate IFNγ production following infection, which likely contributes significantly to their heightened susceptibility to *T. gondii*.

The protective effect of immune serum observed in this study might be at least partially caused by co-transferred IFNγ and TNFα, as these cytokines were consistently elevated in the analyzed batches of pre-transfer immune serum. Since the first immune serum transfer was performed *via* intraperitoneal injection one day prior to *T. gondii* infection, peritoneal immune cell populations may have been pre-activated by the transferred proinflammatory cytokines, contributing to the antibody-mediated protection we aimed to achieve. For future experiments, it will be essential to include recipients of IFNγ-spiked control serum to assess the specific impact of proinflammatory cytokines present in the transferred immune serum. However, it should also be noted that neither WT nor LTβR$^{-/-}$ immune serum recipients displayed elevated levels of IFNγ or TNFα on day 3 p.i. compared to control serum recipients, suggesting that the immune serum transfer from day 2 p.i. did not lead to a significant and/or sustained systemic increase of these cytokines. Interestingly, WT immune serum recipients also

showed significantly lower MHCII expression on splenic proinflammatory monocytes/macrophages compared to control serum recipients on day 3 p.i. Since IFNγ is known to activate proinflammatory monocytes/macrophages and induce MHCII expression [93,94], this finding also suggests that there was no substantial systemic activation from the co-transferred IFNγ – however, this observation does not exclude the possibility of activated peritoneal monocytes/macrophages.

Antibodies exert functions beyond neutralization and opsonization, such as complement activation or mediation of antibody-dependent cellular cytotoxicity [95], and provide protection against intracellular pathogens [86,96-99]. And *vice versa*, defects in the B cell compartment cause increased susceptibility to pathogens such as *T. gondii* [66,67,100]. Thus, despite the relatively limited effect of passive immunization observed in this study, it is important to recognize the significant role of humoral immunity in the defense against (obligate) intracellular pathogens, which can substantially contribute to host defense [65]. The literature on immunization against *T. gondii* reflects a similar complexity, with some studies confirming the protective effects of both active [70,87] and passive immunization [67,75,84-86], while others have found no protective benefit and, in some cases, even suppression of the humoral response in immunized recipients [89-91]. One of the key challenges in this field is the wide range of variables – such as inbred mouse strains, parasite strains, routes of infection, and immunization strategies – that complicate the evaluation of immunization efficacy against this intracellular parasite [101,102].

In our study, passive immunization notably reduced specific *T. gondii*-specific antibodies in the serum of WT recipient mice, particularly anti-GRA7 IgM, anti-GRA8 IgM, and anti-GRA8 IgG, compared to WT control serum recipients. A control experiment confirmed the functionality of our passive immunization model, as transferred antibodies were clearly detectable in the serum of uninfected WT mice 24 hours post-transfer. To exclude the possibility that band signal saturation might have obscured differences in anti-ROP1c IgM titers between immune and control serum recipients, we performed additional line blot tests with increasingly diluted serum. However, these tests still revealed no difference (data not shown). The fact that only specific *T. gondii*-specific antibodies were reduced in immune serum recipients, while others remained comparable to control serum recipients, suggests a specific effect rather than a nonspecific suppression of the antibody response, which has been frequently reported as a consequence of *T. gondii* infection as discussed below.

T. gondii has been shown to interfere with humoral immunity by manipulating macrophages to promote immune evasion [103-105], partly by enabling them to act as suppressor cells. These suppressor macrophages inhibit the proliferative response of lymphocytes to antigen stimulation through direct cell-to-cell contact with target cells, causing involution of splenic follicles during the acute phase of infection and nonspecifically suppressing the antibody response in the host [106-109].

This suppressive effect of *T. gondii* varies drastically between inbred strains of mice, which is, for example, present in C57BL/6 mice but absent from BALB/c mice [106,108]. This factor likely contributes to the varying susceptibility of different mouse strains to *T. gondii* and the issue of high experimental complexity [101,102]. However, this parasite-induced macrophage suppression does not explain the observed reduction of specific antibodies in WT immune serum recipients in this study, as no parasites were co-transferred with the immune serum, and both immune and control serum WT recipients were equally exposed to potential *T. gondii*-derived immune regulation after infection. Nevertheless, we cannot rule out the possibility that unknown soluble factors, either directly from *T. gondii* or from infected cells, may have been co-transferred with the immune serum and contributed to the suppression of the antibody response in recipient mice.

Another crucial factor to consider is the antibody dosage in the transferred immune serum. While our control experiment confirmed that an i.p. injection of immune serum successfully transfers *T. gondii*-specific antibodies into the host's circulatory system, the serum transfer model could be further optimized to potentially increase the efficacy of passive immunization. For instance, administering the immune serum intravenously rather than intraperitoneally might enhance its effectiveness, as this method allows the transferred antibodies to bypass hepatic metabolism and directly enter the recipient's circulatory system [110]. Studies have demonstrated that the antibody response to *Toxoplasma gondii* [90] or other pathogens [111,112] can be either enhanced or suppressed in passive immunization experiments, with the antigen-to-antibody ratio being a key contributing factor to this variation. However, while too few antibodies may fail to provide adequate protection, excessively high doses might also adversely affect the humoral immune response [111]. In general, there are several proposed mechanisms that may explain how transferred antibodies can interfere with the endogenous antibody production in immune serum recipient mice [113,114]. One such mechanism is epitope masking, where the transferred antibodies bind to specific *T. gondii* epitopes, preventing the immune system from recognizing and targeting them effectively, leading to lower overall antibody levels against those epitopes in the recipients.

Importantly, different immunoglobulin isotypes have varying effects on the antibody response in recipients. While both transferred IgM and IgG can either suppress or enhance immune responses, IgM is more commonly associated with enhancing antibody production. Conversely, IgG, especially at high doses or with high affinity, is well-known for its ability to suppress antibody responses, with all IgG subclasses being capable of this effect [113,115]. The observed reduction in some but not all analyzed *T. gondii*-specific antibodies in WT immune serum recipients may be attributed to the transferred IgG antibodies. Specifically, IgM against GRA7 and GRA8 was significantly lower in immune serum recipients compared control serum recipients, while anti-ROP1c IgM levels remained relatively comparable. This discrepancy could be explained by the lower levels of IgG directed against ROP1c

compared to GRA7 and GRA8 in the pre-transfer immune serum. The transferred IgG antibodies may have interfered with the production of endogenous IgM against GRA7 and GRA8, while their impact on ROP1c was limited due to the smaller amounts of transferred anti-ROP1c IgG. In line with this hypothesis, transferred anti-ROP1c IgG was undetectable in the serum of uninfected mice 24 hours post-transfer in the control experiment. In contrast, anti-GRA7 IgG was clearly detectable, and anti-GRA8 IgG was weakly detectable, confirming lower systemic levels of transferred anti-ROP1c IgG in recipient mice.

However, the precise mechanisms underlying IgG-mediated antibody suppression are still not fully understood. In addition to epitope masking, trogocytosis has been suggested as a possible mechanism [115]. Trogocytosis refers to the process where lymphocytes capture membrane fragments from antigen-presenting cells (APCs) during immunological synapse formation [116]. In this context, the binding of transferred IgG to antigens on the surface of APCs may trigger the removal of these antigens through trogocytosis, thereby reducing their availability for recognition by the host's immune system and impairing the subsequent antibody response. Other potential mechanisms include Fcγ receptor (FcγR)-mediated B cell inhibition, activation of the complement system, and increased clearance of IgG-antigen complexes [115]. Our results suggest that while the transfer of pre-existing IgG antibodies did not diminish the overall benefit of the immune serum – evidenced by the prolonged survival of LTβR$^{-/-}$ mice – it can specifically suppress certain components of the antibody response, such as the endogenous production of anti-GRA7 and anti-GRA8 IgM. This selective suppression is an important consideration for potential therapeutic applications.

Impact of LTβR deletion in myeloid cells on survival following *T. gondii* infection

In addition to our investigation of complete LTβR-deficient mice, we aimed to investigate the impact of specifically targeted LTβR inactivation in myeloid (LTβR-LysMCre) and dendritic (LTβR-CD11c-Cre) cells on mouse survival during *T. gondii* infection. LTβR-CreDeleter mice served as positive controls for ubiquitous Cre/loxP-mediated LTβR inactivation. As expected, these mice displayed a high susceptibility to *T. gondii* infection and completely succumbed during the acute stage, comparable to LTβR$^{-/-}$ mice. LTβR$^{fl/fl}$ mice served as negative controls and similar to WT mice, they survived the acute phase but began to succumb during the early chronic stage, primarily due to progressive weight loss.

Notably, all investigated mouse strains displayed significantly prolonged survival compared to LTβR$^{-/-}$ and LTβR-CreDeleter mice, confirming the increased susceptibility of these strains to *T. gondii*. LTβR-LysMCre exhibited a tendency towards reduced survival, especially during the acute stage of infection, compared to WT mice. This reduction in survival was statistically significant when compared to LTβR-

CD11cCre survival. Therefore, the question arises as to how the absence of LTβR signaling in myeloid cells and DCs might affect the immune response against *T. gondii*.

The role of LTβR signaling in myeloid cells remains ambiguous. LTβR signaling in both macrophages [2] and neutrophils [3] has been shown to ameliorate otherwise exacerbated DSS-induced colitis, indicating a role in controlling and down-regulating the inflammatory response [117,118]. In line with these findings, other studies showed that LTβR signaling in macrophages does not induce proinflammatory cytokine expression but rather promotes expression of TRIM30α, a regulator of NF-κB activation [119], rendering macrophages hyporesponsive to subsequent TLR-stimulation [1,120]. However, IL-8 is a notable exception, as its expression is enhanced by LTβR signaling in human macrophage-like THP-1 cells [121] and bronchial epithelial cells [122]. Furthermore, LTβR activation on murine bone marrow-derived mast cells induces the expression of cytokines such as IL-4, IL-6 and TNFα, indicating a proinflammatory role instead [123].

Even less is known about the impact of LTβR deficiency in myeloid cells during actual infection challenges. One study found that while LTβR deficiency in myeloid cells leads to increased bacterial loads in blood and feces, the mice were still able to survive *Citrobacter rodentium* infection, suggesting a mild to moderate immunological deficiency [124]. Similarly, the observed trend towards reduced survival in LTβR-LysMCre mice compared to WT mice in this study indicates a potential negative effect of LTβR inactivation in myeloid cells on mouse survival, though whether this difference reaches statistical significance has yet to be determined. If significant, the loss of LTβR signaling in myeloid cells could potentially lead to pro-inflammatory effects (e.g. exacerbated inflammation, immunopathology) or anti-inflammatory effects (e.g. loss of function, reduced cytokine expression) and/or changes in migratory responses, ultimately contributing to the observed reduction in survival.

In contrast, LTβR-CD11cCre mice did not exhibit increased susceptibility to *T. gondii* infection. All mice survived the acute stage and showed even a trend towards prolonged survival compared to WT mice in the chronic stage. LTβR signaling is required for the development, homeostasis, and function of DCs [4,11,13,91,125]. During *T. gondii* infection, DCs, particularly CD8α$^+$ DCs, play a critical role in the detection of parasite-derived profilin and serve as major producers of IL-12 [49,126,127]. Interestingly, lymphoid tissue CD8α$^+$ DCs are less affected by the absence of LTβR signaling than other DC subpopulations [4], which could explain the unaffected survival of LTβR-CD11cCre mice. Additionally, *T. gondii* preferentially infects DCs and may exploit them as a Trojan horse for dissemination to immune-privileged organs, such as the brain [128-130]. This shuttle mechanism could be impaired in LTβR-CD11cCre mice, potentially contributing to prolonged survival during the chronic stage.

All mouse lines investigated exhibited severe weight loss, which temporarily stabilized during the transition between the acute and chronic stages of infection. While all remaining lines (WT, LTβR-

LysMCre, LTβR-CD11cCre, and LTβR$^{fl/fl}$) displayed improved activity and grooming in the chronic stage, their weight loss continued, ultimately requiring the euthanasia and removal of several mice from the experiment. *T. gondii* infection is known to cause severe intestinal inflammation and alterations to the gut microbiome, with C57BL/6 mice being more susceptible compared to genetically resistant BALB/c mice [131-135]. It is therefore conceivable that unresolved intestinal inflammation may contribute to the continued weight loss during the chronic stage of infection.

Notably, the tendency towards increased susceptibility of LTβR-LysMCre mice during the acute stage was biased to male mice, whereas female mice started to succumb during the early chronic stage. Additionally, male WT mice showed a tendency for prolonged survival compared to females during the chronic stage, suggesting potential sex-based differences in susceptibility to *T. gondii* infection. In literature, sex-determined differences in the murine immune response against *T. gondii* vary depending on factors such as the mouse strain, route of infection, and parasite strain (mainly type I or II) used. For example, female SCID mice infected orally with the Beverly type II strain displayed a delayed upregulation of IL-12 and reduced levels of IFNγ in the serum compared to male mice [136]. In contrast, female BALB/c mice infected i.p. with the Prugniaud type II strain displayed significantly increased serum concentrations of IFNγ (but not IL-12p70), TNFα and MCP-1 compared to male mice [137]. In another study, female CBA/J mice infected i.p. with the ME49 type II strain also showed a tendency for increased IFNγ, IL-6 and MCP-1 concentrations during chronic infection compared to male mice, though this difference was not statistically significant [138]. Interestingly, female mice have more numerous tissue-resident leukocyte populations, including macrophages, which respond more efficiently to acute inflammation due to greater TLR-expression, enhanced phagocytosis and NADPH-oxidase-mediated pathogen elimination [139]. At the same time, this is balanced by a higher presence of immunomodulatory CD4^{+} T cells [139]. If LTβR deficiency impairs myeloid cell functionality in LTβR-LysMCre mice, this could be particularly detrimental to male mice due to their potentially less efficient acute immune response, contributing to their higher mortality during the acute stage of *T. gondii* infection.

However, in another study, an investigation of the transcriptome of male and female CBA/J mice during *T. gondii* (ME49) infection did not find a profound sexual dimorphism, as both sexes displayed an overall comparable immune response and no significant difference in their survival rates [138]. Although male mice expressed greater amounts of low-abundance genes and had more brain cysts than female mice at three months p.i., this difference in cyst numbers was no longer observed at six months p.i.

In conclusion, while there may be certain sex-determined differences during the immune response to *T. gondii*, these differences may also be influenced by variables such as mouse strains (genetic

backgrounds as well as specific alterations, such as the LTβR inactivation in myeloid cells in LTβR-LysMCre mice), parasite strains, and routes of infection. As sexual dimorphism is increasingly recognized in infectious diseases and in the concept of personalized medicine [140], it is important to consider this variable in future experiments regarding antibody-mediated protection against *T. gondii* as well as the role of LTβR signaling.

Materials and Methods

Mice. LTβR$^{-/-}$ mice were described previously [12] and back-crossed to a C57BL6/N background for at least 10 generations. Wildtype (WT) littermates were used as controls. Mice were housed in the animal facility of the Heinrich Heine University Düsseldorf under specific-pathogen-free (SPF) conditions and were 8 to 16 weeks old at the time of infection; both male and female mice were used. CD1 mice from Charles River Breeding Laboratories were used to maintain and propagate ME49 *Toxoplasma gondii* for infection experiments. IFNγR$^{-/-}$ mice were used as infection controls. For the survival experiments with Cre/loxP-mediated, cell type-specific LTβR inactivation, LTβR-LysMCre, LTβR-CD11cCre, and LTβR-CreDeleter mice were generated by crossing LTβR-floxed mice (LTβR$^{fl/fl}$, [124]) with LysM-Cre [141], CD11c-Cre [142], and Cre-Deleter [143] mice, respectively. CD11c-Cre (B6J) mice were a gift of Prof. Ingo Drexler and used to generate the LTβR$^{fl/fl}$ x CD11c-Cre line, which was back-crossed to the LTβR$^{fl/fl}$ (B6N) line for at least three generations. All animal experiments were conducted in strict accordance with the German Animal Welfare Act. The protocols were approved by the local authorities (Permit no. 81-02.04.2018.A406, 81-02.04.2021.A060 and 81-02.05.40.18.082). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

T. gondii **cyst preparation.** ME49 (type II strain) cysts were isolated from the brains of CD1 mice *via* Ficoll-Paque gradient centrifugation 11 to 19 weeks after infection. Briefly, the murine cerebrum was homogenized by passaging through successively thinner cannulas (smallest size: 23G). After centrifugation (130 x g, 5 min, room temperature (RT)), the pellet was resuspended in 20 ml PBS. 10 ml of Ficoll-Paque Plus (GE Healthcare, USA) was carefully layered below the PBS, followed by centrifugation (1,250 x g, 25 min, RT) without brakes. Pelleted cysts were washed with PBS, counted (10 cysts per mouse) and lysed with Trypsin-EDTA. Lysis was stopped with the addition of heat-inactivated (56 °C, 30 min) fetal calf serum (FCS, PAN-Biotech, Germany). After a final wash with PBS followed by centrifugation (660 x g, 10 min, RT), ME49 bradyzoites were resuspended in 0.2 ml PBS per murine recipient.

Generation of control and immune serum. Uninfected WT mice aged between 8 and 16 weeks were anesthetized with 100 mg/kg Ketamine (Zoetis, USA) and 10 mg/kg Xylazine (Elanco, USA), and then

bled through the *vena cava inferior* (20G cannula). The blood was allowed to coagulate for 30 min at room temperature (RT), centrifuged twice (10 min, 8,000 x g), and pooled to generate control serum. To generate immune serum, the same procedure was followed using *T. gondii* infected WT mice (ME49, 10 cysts) 14 days after infection. 4 batches of immune serum were analyzed for *T. gondii*-specific IgM and IgG antibodies *via* serial dilution and line blot assay, with control serum serving as negative control.

Detection of *T. gondii*-specific antibodies. *T. gondii*-specific IgM and IgG serum antibodies were detected with line blots loaded with recombinant *T. gondii*-antigens (RecomLine Toxoplasma IgM/IgG, Mikrogen, Germany). IgM and IgG conjugates (anti-human) provided within the kit were replaced with anti-mouse IgM-HRP and IgG-HRP antibodies (Thermo Fisher Scientific, USA) to allow assessment of murine samples. While the line blot IgM controls functioned with this adjustment, the IgG controls and cut-off controls did not work with murine samples. The assay was performed according to the manufacturer's protocol, and donor control and immune serum served as negative and positive controls, respectively. Band signal intensities of line blot images (TIFF format, 600 dpi, grayscale) were determined as grey mean values using FIJI/ImageJ software and normalized to the values of pre-transfer WT donor immune serum. The WT donor immune serum itself was semi-quantified by a range of serial dilutions (1:100, 1:400, 1:1,600, 1:6,400, 1:12,800 and 1:25,600) before line blot analysis.

Serum transfer. Acceptor WT and LTβR$^{-/-}$ mice received 0.2 ml of undiluted control or immune serum via intraperitoneal injection one day prior to infection (d-1) and on days 3, 6 and 9 p.i. As control, immune serum was transferred once *via* i.p. injection into two uninfected WT mice, which were then sacrificed and bled 24 hours later. The generated serum was then analyzed for transferred *T. gondii*-specific antibodies *via* line blot assay.

***T. gondii* infection in mice.** Age- and sex-matched mice, 8 to 16 weeks old, were infected with *T. gondii* bradyzoites (ME49, from 10 cysts per murine recipient) in 0.2 ml PBS via i.p. injection and were weighed and scored daily for the duration of the experiment.

Mouse material preparation and processing. The mice were anesthetized with 100 mg/kg Ketamine (Zoetis, USA) and 10 mg/kg Xylazine (Elanco, USA). **Peritoneal lavage**: was performed by injection of 5 ml ice-cold PBS (22G cannula) into the peritoneal cavity, followed by a gentle massage and peritoneal fluid extraction. Aliquots were prepared for flow cytometry and quantitative real-time PCR (qRT-PCR). For qRT-PCR, the extracted fluid was centrifuged (470 x g, 3 min) and total DNA was isolated from the remaining pellet using a DNA isolation kit (Genekam, Germany) according to the manual. Samples were adjusted to 100 ng/µl DNA. **Spleen**: The spleen was harvested, weighed, and a part of the caudoventral extremity was cut and removed for qRT-PCR. For qRT-PCR, the tissue was homogenized with Precelly beads (CK28) and the Precelly Evolution Homogenizer (2x 5,500 RPM for 20 sec; Bertin, France) prior

to DNA isolation. For flow cytometry, remaining spleen tissue was shredded and incubated for 30 min at 37 °C in digestion buffer (PBS + 3,000 U DNAse I/ml [Thermo Fisher Scientific, USA] + 1 mg/ml Collagenase [Sigma-Aldrich, USA]). After the addition of PBS + 10 mM EDTA, splenocytes were filtered through a 70 µm strainer, centrifuged (470 x g, 3 min, 4 °C) and treated with red blood cell lysis buffer (Morphisto, Germany). Remaining leukocytes were washed with PBS, counted, and stained for flow cytometry. **Lung** and **brain:** The left lung and brain tissue were harvested and prepared for qRT-PCR as described for the spleen.

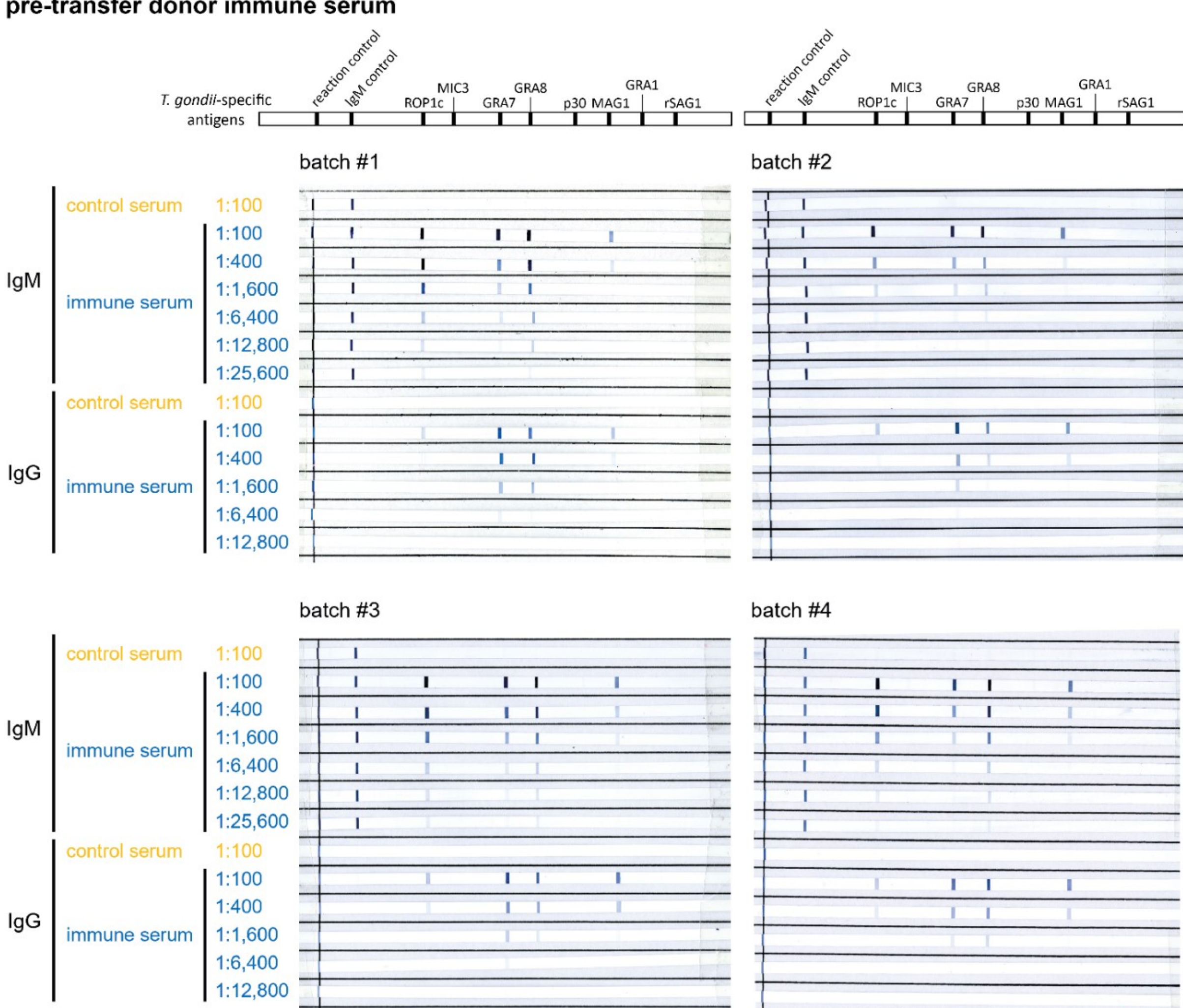
Detection of parasite burden *via* qRT-PCR. *TgB1* primer (Forward: 5'-GCT AAA GGC GTC ATT GCT GTT-3', Reverse: 5'-GGC GGA ACC AAC GGA AAT-3') and a FAM-probe (5'→3': FAM-ATC GCA ACG GAG TTC TTC CCA GAC GT-BHQ1), both purchased from Metabion (Germany) were used to amplify and detect a defined sequence of the 35-fold repetitive B1 gene from *T. gondii*. qRT-PCR was performed on Bio-Rad CFX-96 systems. DNA isolated from a defined number of ME49 tachyzoites (2.914 ± 214/µl) was used to generate a standard curve and calculate parasite loads.

Cell surface staining for flow cytometry. Cells were stained in U-bottom polystyrene plates and were incubated with Fc-blocking CD16/32 antibody (1:100) in staining buffer (1x PBS, 2 mM EDTA, 2% FCS (v/v)) for 30 min at 4 °C. Fluorophore-labeled antibodies were prepared in staining buffer, added to cells (final volume: 50 µl) and incubated for 30 min (4 °C, light-protected). Cells were then washed and analyzed with a BD LSRFortessa II flow cytometer and FlowJo v10.8 software. All antibodies and dyes are listed in Supplementary Table 1, all cell populations are listed in Supplementary Table 2, and all gating strategies are shown in Supplementary Figures 4 & 5.

Cytokine concentrations. Murine cytokines were measured with a pre-selected bead-based multiplex panel (LEGENDplex Mouse Inflammation Panel from BioLegend, USA) according to the manufacturer's protocol. Samples were acquired with a BD FACSCanto II or LSRFortessa II and analyzed with the Qognit software (BioLegend).

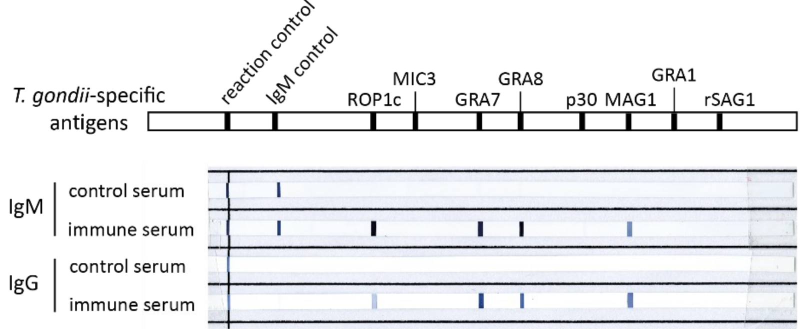
Statistical analysis. GraphPad Prism software (version 10) was used for data analysis. Symbols represent individual animals, columns represent mean values, and error bars represent the ± standard deviation (SD). Outliers were identified and excluded from the data using the ROUT test (Q = 1%) after which the Shapiro-Wilk test of normality was performed. Parametric data was analyzed using 2-way analysis of variance (ANOVA) corrected for multiple comparison using Tukey's *post hoc* test. Nonparametric data was analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Comparisons with a *P* value of ≥ 0.05 were considered statistically not significant and were not specifically marked.

Supplementary information

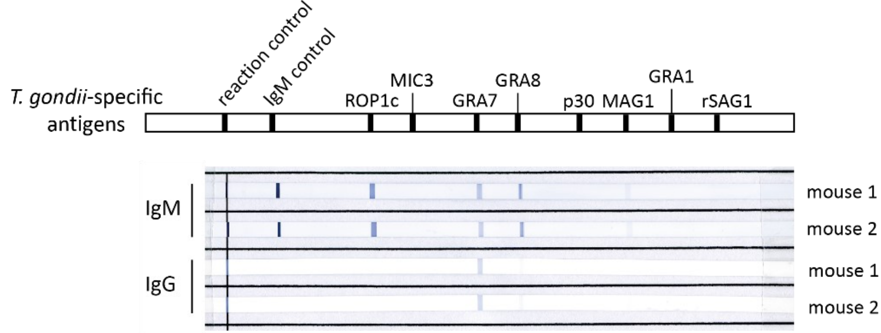


Supplementary Figure 1: Semi-quantification of *T. gondii*-specific antibodies in the four pre-transfer WT donor immune serum batches *via* serial dilution. *T. gondii*-specific IgM and IgG serum antibodies were quantified using commercially available line blots loaded with recombinant *T. gondii* antigens (Recomline, Mikrogen, Germany).

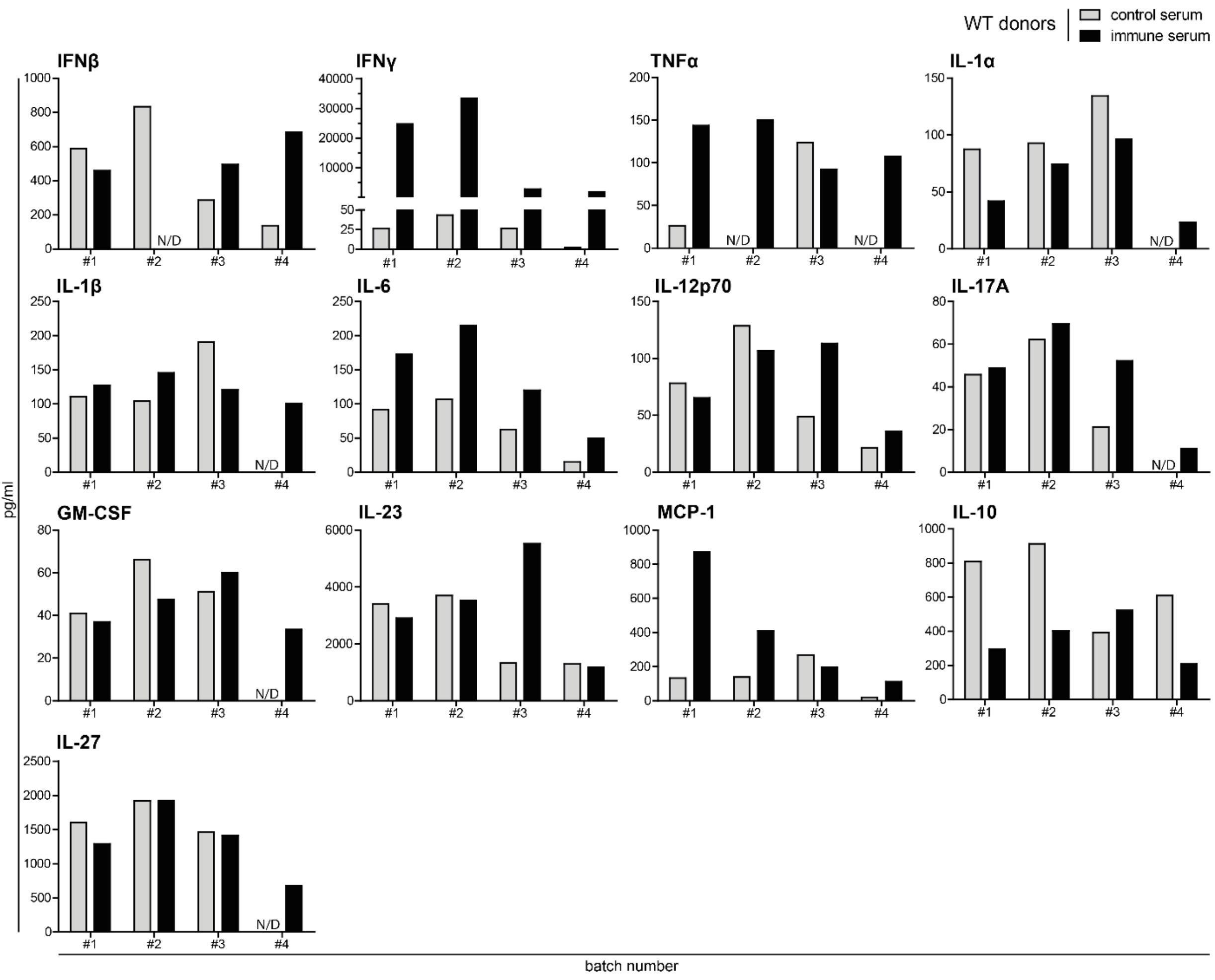
pre-transfer donor immune serum



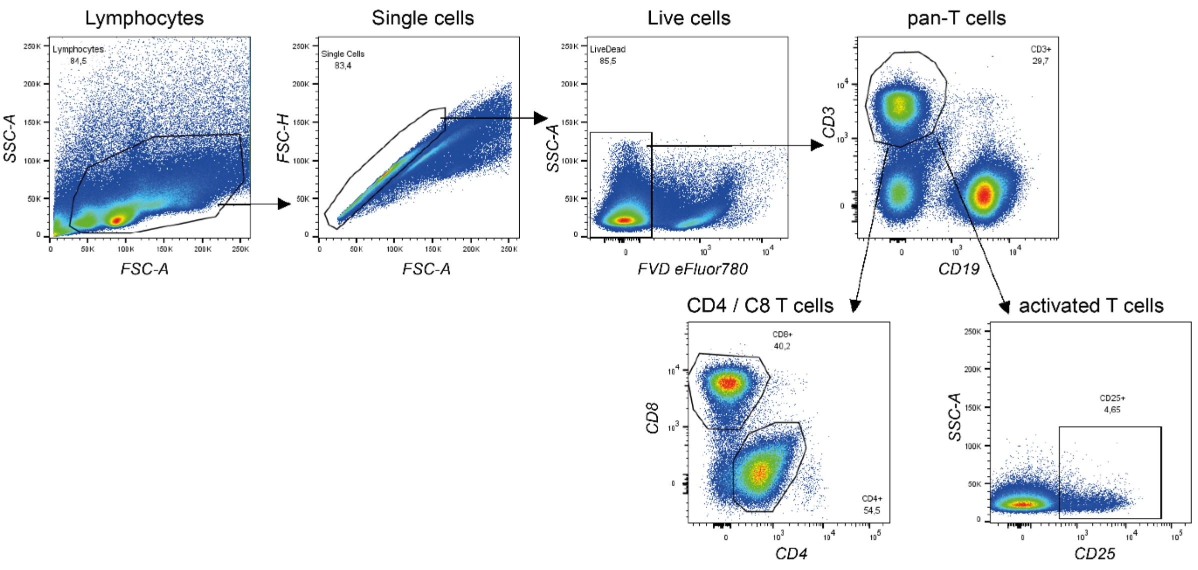
recipient serum 24h post-transfer



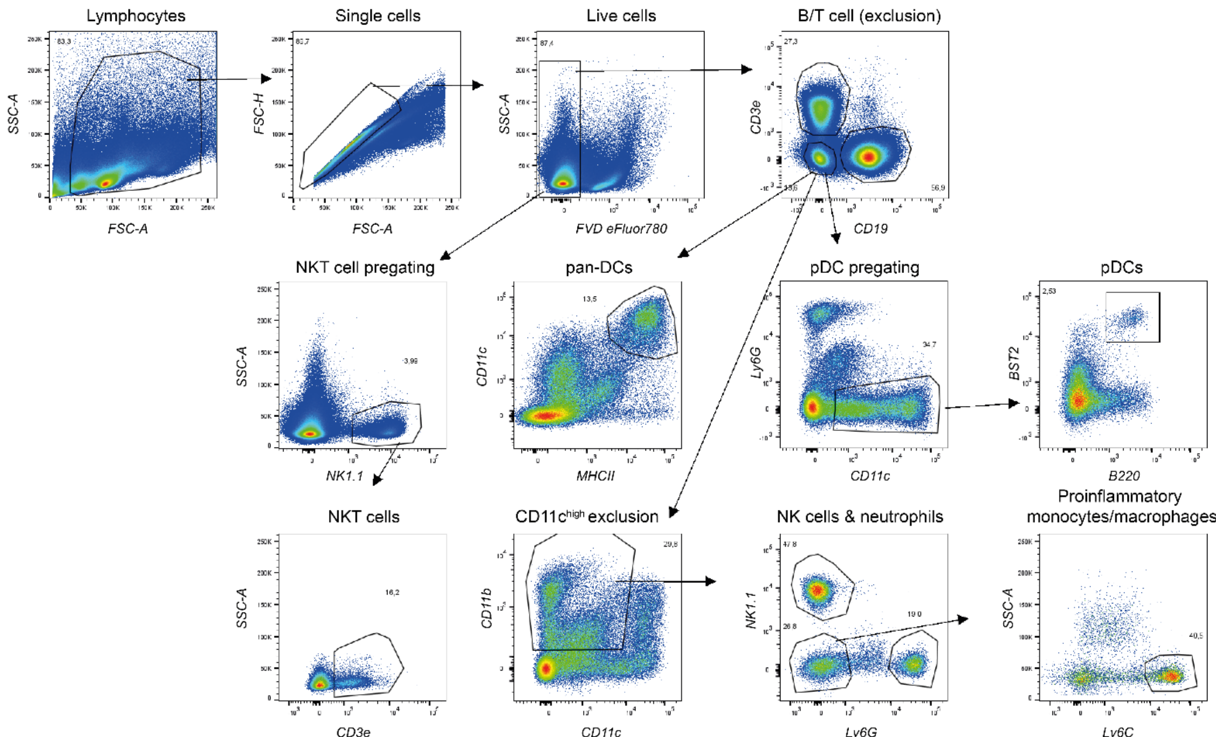
Supplementary Figure 2: Transferred *T. gondii*-specific antibodies are detectable in the serum of recipient mice. Pooled donor immune and control sera were analyzed pre-transfer for *T. gondii*-specific IgM and IgG using antigen-loaded line blots (Recomline, Mikrogen, Germany). The immune serum was then injected once into two uninfected WT mice (200 µl, i.p.). After 24 hours the sera of these uninfected mice were analyzed for the presence of the transferred *T. gondii*-specific IgM and IgG using the same method.



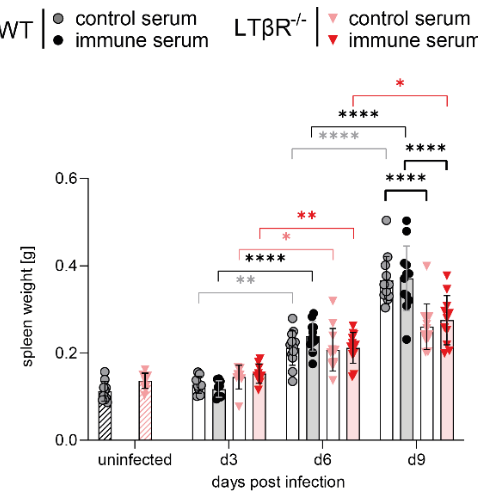
Supplementary Figure 3: Cytokine profiles in the four batches of pre-transfer WT donor immune and control serum. Cytokine concentrations were measured using a bead-based multiplex assay (LegendPlex, BioLegend, USA).



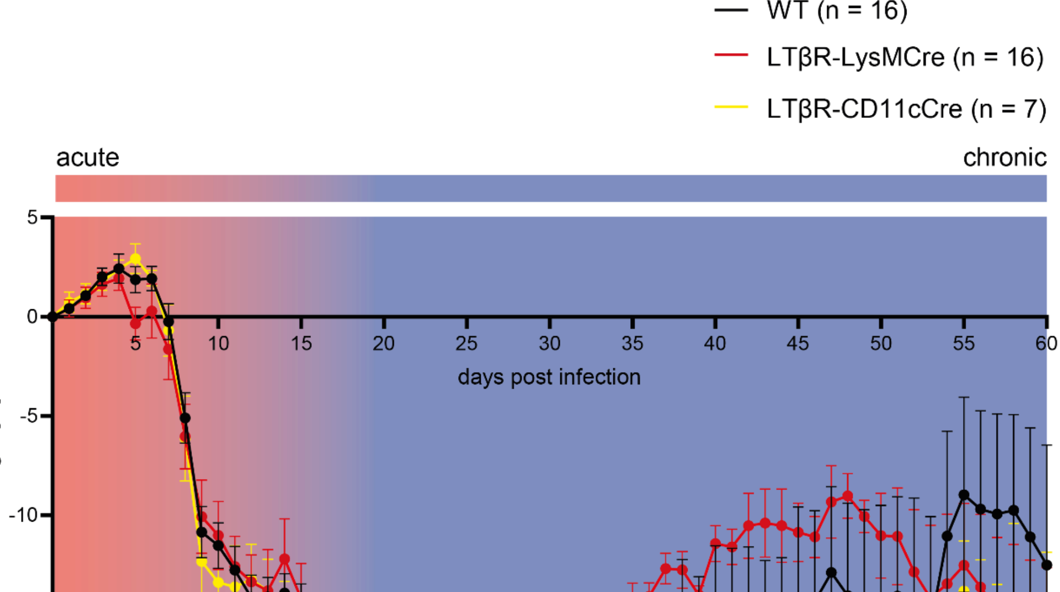
Supplementary Figure 4: Gating strategy for the identification of murine T cell populations in the spleen.



Supplementary Figure 5: Gating strategy for the identification of murine B cells, NK cells, NKT cells, pan-DCs, pDCs, neutrophils, proinflammatory monocytes/macrophages in the spleen.



Supplementary Figure 6: Passive immunization does not influence spleen weight in WT and LTβR$^{-/-}$ mice. All data shown represent at least four independent experiments; symbols represent individual animals and columns represent means ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.



Supplementary Figure 7: WT, LTβR-LysMCre and LTβR-CD11cCre mice show comparable weight loss during *T. gondii* infection. Relative weight loss during infection is expressed as a percentage change from baseline (day 0) weight.

Antibodies	Clone	Fluorochromes	Final dilution	Manufacturer
BST-2 (CD317, PDCA1)	927	BV650	1:150	BioLegend
B220 (CD45R)	RA3-6B2	FITC	1:150	BD Biosciences
CD3e	145-2C11	BV711	1:150	BioLegend
CD3e	145-2C11	FITC	1:150	BD Biosciences
CD4	GK1.5	PerCP-Cy5.5	1:200	BioLegend
CD8a	53/6.7	APC	1:150	BD Biosciences
CD11b	M1/70	AlexaFluor 700	1:200	BioLegend
CD11c	HL3	PE-Cy7	1:200	BD Biosciences
CD16/32	93	-	1:100	eBioscience
CD19	6D5	BV785	1:150	BioLegend
CD25	PC61	PE-Cy7	1:150	BioLegend
Ly6C	HK1.4	BV605	1:200	BioLegend
Ly6G	1A8	PerCP-Cy5.5	1:200	BioLegend
MHCII (I-A/I-E)	M5/114.15.2	BV510	1:200	BioLegend
NK1.1	PK136	BV421	1:200	BioLegend
-	-	Fixable Viability Dye (FVD) eFluor780	1:5000	eBioscience

Supplementary Table 1: Antibody-conjugates and dyes utilized in the flow cytometry analysis. Final dilution refers to antibody dilution after addition to cells incubated in Fc-block (CD16/32) solution. APC = Allophycocyanin,

BV = Brilliant Violet, Cy = Cyanine, FITC = Fluorescein, PE = Phycoerythrin, PerCP = Peridinin-Chlorophyll-protein.

Immune cell population	**Marker**	**Gating strategy supplementary figure**
live cells	FVD eFluor780-	Suppl. Fig. 4 & 5
B cells	CD3- CD19+	Suppl. Fig. 4 & 5
pan-T cells	CD3+ CD19-	Suppl. Fig. 4 & 5
CD4 T cells	CD19- CD3+ CD4+	Suppl. Fig. 4
CD8 T cells	CD19- CD3+ CD8+	Suppl. Fig. 4
activated T cells	CD19- CD3+ CD25+	Suppl. Fig. 4
NKT cells	CD19- CD3+ NK1.1+	Suppl. Fig. 5
pan-DCs	CD3- CD19- NK1.1- CD11c+ MHCII+	Suppl. Fig. 5
pDCs	CD3- CD19- NK1.1- CD11c+ B220+ BST2+	Suppl. Fig. 5
NK cells	CD3- CD19- $CD11c^{high}$- CD11b+ NK1.1+ Ly6G-	Suppl. Fig. 5
Neutrophils	CD3- CD19- $CD11c^{high}$- CD11b+ NK1.1- Ly6G+	Suppl. Fig. 5
Proinflammatory monocytes/macrophages	CD3- CD19- $CD11c^{high}$- CD11b+ NK1.1- Ly6G- Ly6C+	Suppl. Fig. 5

Supplementary Table 2: Selected murine splenic immune cell populations and surface markers and dyes used for their identification.

Data availability

The data that support the findings of this study are available from the corresponding author.

Acknowledgements

We thank Nicole Küpper, Julia Mock and Sonja Schavier for technical assistance.

This work was supported by the Jürgen Manchot Foundation (Molecules of Infection IV – MOI IV).

Author contributions

M.H. performed all experiments, with support from U.R.S. M.H. analyzed and visualized all data shown. M.H., U.R.S. and K.P. designed the study and wrote the manuscript with input from D.D.

Literature:

1. Wimmer, N., Huber, B., Barabas, N., Rohrl, J., Pfeffer, K., and Hehlgans, T. (2012). *Lymphotoxin beta receptor activation on macrophages induces cross-tolerance to TLR4 and TLR9 ligands*. J Immunol ***188***, 3426-3433. 10.4049/jimmunol.1103324.

2. Wimmer, N., Huber, B., Wege, A.K., Barabas, N., Rohrl, J., Pfeffer, K., and Hehlgans, T. (2012). *Lymphotoxin-beta receptor activation on macrophages ameliorates acute DSS-induced intestinal inflammation in a TRIM30alpha-dependent manner*. Mol Immunol ***51***, 128-135. 10.1016/j.molimm.2012.02.118.

3. Riffelmacher, T., Giles, D.A., Zahner, S., Dicker, M., Andreyev, A.Y., McArdle, S., Perez-Jeldres, T., van der Gracht, E., Murray, M.P., Hartmann, N., Tumanov, A.V., and Kronenberg, M. (2021). *Metabolic activation and colitis pathogenesis is prevented by lymphotoxin beta receptor expression in neutrophils*. Mucosal Immunol ***14***, 679-690. 10.1038/s41385-021-00378-7.

4. Kabashima, K., Banks, T.A., Ansel, K.M., Lu, T.T., Ware, C.F., and Cyster, J.G. (2005). *Intrinsic lymphotoxin-beta receptor requirement for homeostasis of lymphoid tissue dendritic cells*. Immunity ***22***, 439-450. 10.1016/j.immuni.2005.02.007.

5. Ware, C.F. (2005). *Network communications: lymphotoxins, LIGHT, and TNF*. Annu Rev Immunol ***23***, 787-819. 10.1146/annurev.immunol.23.021704.115719.

6. Hehlgans, T., and Pfeffer, K. (2005). *The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games*. Immunology ***115***, 1-20. 10.1111/j.1365-2567.2005.02143.x.

7. Remouchamps, C., Boutaffala, L., Ganeff, C., and Dejardin, E. (2011). *Biology and signal transduction pathways of the Lymphotoxin-alphabeta/LTbetaR system*. Cytokine Growth Factor Rev ***22***, 301-310. 10.1016/j.cytogfr.2011.11.007.

8. Ward-Kavanagh, L.K., Lin, W.W., Sedy, J.R., and Ware, C.F. (2016). *The TNF Receptor Superfamily in Co-stimulating and Co-inhibitory Responses*. Immunity ***44***, 1005-1019. 10.1016/j.immuni.2016.04.019.

9. Dejardin, E., Droin, N.M., Delhase, M., Haas, E., Cao, Y., Makris, C., Li, Z.W., Karin, M., Ware, C.F., and Green, D.R. (2002). *The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways*. Immunity ***17***, 525-535. 10.1016/s1074-7613(02)00423-5.

10. Norris, P.S., and Ware, C.F. (2007). *The LT beta R signaling pathway*. Adv Exp Med Biol ***597***, 160-172. 10.1007/978-0-387-70630-6_13.

11. Shou, Y., Koroleva, E., Spencer, C.M., Shein, S.A., Korchagina, A.A., Yusoof, K.A., Parthasarathy, R., Leadbetter, E.A., Akopian, A.N., Munoz, A.R., and Tumanov, A.V. (2021). *Redefining the Role of Lymphotoxin Beta Receptor in the Maintenance of Lymphoid Organs and Immune Cell Homeostasis in Adulthood*. Front Immunol ***12***, 712632. 10.3389/fimmu.2021.712632.

12. Futterer, A., Mink, K., Luz, A., Kosco-Vilbois, M.H., and Pfeffer, K. (1998). *The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues*. Immunity ***9***, 59-70.

13. Wu, Q., Wang, Y., Wang, J., Hedgeman, E.O., Browning, J.L., and Fu, Y.X. (1999). *The requirement of membrane lymphotoxin for the presence of dendritic cells in lymphoid tissues*. J Exp Med ***190***, 629-638. 10.1084/jem.190.5.629.

14. Elewaut, D., Brossay, L., Santee, S.M., Naidenko, O.V., Burdin, N., De Winter, H., Matsuda, J., Ware, C.F., Cheroutre, H., and Kronenberg, M. (2000). *Membrane lymphotoxin is required for the development of different subpopulations of NK T cells*. J Immunol ***165***, 671-679. 10.4049/jimmunol.165.2.671.

15. Franki, A.S., Van Beneden, K., Dewint, P., Hammond, K.J., Lambrecht, S., Leclercq, G., Kronenberg, M., Deforce, D., and Elewaut, D. (2006). *A unique lymphotoxin alphabeta-dependent pathway regulates thymic emigration of Valpha14 invariant natural killer T cells*. Proc Natl Acad Sci U S A ***103***, 9160-9165. 10.1073/pnas.0508892103.

16. Iizuka, K., Chaplin, D.D., Wang, Y., Wu, Q., Pegg, L.E., Yokoyama, W.M., and Fu, Y.X. (1999). *Requirement for membrane lymphotoxin in natural killer cell development*. Proc Natl Acad Sci U S A ***96***, 6336-6340. 10.1073/pnas.96.11.6336.

17. Tersteegen, A., Sorg, U.R., Virgen-Slane, R., Helle, M., Petzsch, P., Dunay, I.R., Kohrer, K., Degrandi, D., Ware, C.F., and Pfeffer, K. (2021). *Lymphotoxin beta Receptor: a Crucial Role in Innate and Adaptive Immune Responses against Toxoplasma gondii*. Infect Immun ***89***. 10.1128/IAI.00026-21.

18. Borelli, A., and Irla, M. (2021). *Lymphotoxin: from the physiology to the regeneration of the thymic function*. Cell Death Differ ***28***, 2305-2314. 10.1038/s41418-021-00834-8.

19. Fu, Y.X., Huang, G., Wang, Y., and Chaplin, D.D. (1998). *B lymphocytes induce the formation of follicular dendritic cell clusters in a lymphotoxin alpha-dependent fashion*. J Exp Med ***187***, 1009-1018. 10.1084/jem.187.7.1009.

20. Ngo, V.N., Korner, H., Gunn, M.D., Schmidt, K.N., Riminton, D.S., Cooper, M.D., Browning, J.L., Sedgwick, J.D., and Cyster, J.G. (1999). *Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen*. J Exp Med ***189***, 403-412. 10.1084/jem.189.2.403.

21. Vu, F., Dianzani, U., Ware, C.F., Mak, T., and Gommerman, J.L. (2008). *ICOS, CD40, and lymphotoxin beta receptors signal sequentially and interdependently to initiate a germinal center reaction*. J Immunol ***180***, 2284-2293. 10.4049/jimmunol.180.4.2284.

22. Zindl, C.L., Kim, T.H., Zeng, M., Archambault, A.S., Grayson, M.H., Choi, K., Schreiber, R.D., and Chaplin, D.D. (2009). *The lymphotoxin LTalpha(1)beta(2) controls postnatal and adult spleen marginal sinus vascular structure and function*. Immunity ***30***, 408-420. 10.1016/j.immuni.2009.01.010.

23. Myers, R.C., King, R.G., Carter, R.H., and Justement, L.B. (2013). *Lymphotoxin alpha1beta2 expression on B cells is required for follicular dendritic cell activation during the germinal center response*. Eur J Immunol ***43***, 348-359. 10.1002/eji.201242471.

24. Camara, A., Lavanant, A.C., Abe, J., Desforges, H.L., Alexandre, Y.O., Girardi, E., Igamberdieva, Z., Asano, K., Tanaka, M., Hehlgans, T., Pfeffer, K., Pfeffer, S., Mueller, S.N., Stein, J.V., and Mueller, C.G. (2022). *CD169(+) macrophages in lymph node and spleen critically depend on dual RANK and LTbetaR signaling*. Proc Natl Acad Sci U S A ***119***. 10.1073/pnas.2108540119.

25. Banks, T.A., Rickert, S., and Ware, C.F. (2006). *Restoring immune defenses via lymphotoxin signaling: lessons from cytomegalovirus*. Immunol Res ***34***, 243-254. 10.1385/IR:34:3:243.

26. Behnke, K., Sorg, U.R., Gabbert, H.E., and Pfeffer, K. (2017). *The Lymphotoxin beta Receptor Is Essential for Upregulation of IFN-Induced Guanylate-Binding Proteins and Survival after Toxoplasma gondii Infection*. Mediators Inflamm ***2017***, 7375818. 10.1155/2017/7375818.

27. Ehlers, S., Holscher, C., Scheu, S., Tertilt, C., Hehlgans, T., Suwinski, J., Endres, R., and Pfeffer, K. (2003). *The lymphotoxin beta receptor is critically involved in controlling infections with the intracellular pathogens Mycobacterium tuberculosis and Listeria monocytogenes*. J Immunol ***170***, 5210-5218.

28. Jin, L., Guo, X., Shen, C., Hao, X., Sun, P., Li, P., Xu, T., Hu, C., Rose, O., Zhou, H., Yang, M., Qin, C.F., Guo, J., Peng, H., Zhu, M., Cheng, G., Qi, X., and Lai, R. (2018). *Salivary factor LTRIN from Aedes aegypti facilitates the transmission of Zika virus by interfering with the lymphotoxin-beta receptor*. Nat Immunol ***19***, 342-353. 10.1038/s41590-018-0063-9.

29. Puglielli, M.T., Browning, J.L., Brewer, A.W., Schreiber, R.D., Shieh, W.J., Altman, J.D., Oldstone, M.B., Zaki, S.R., and Ahmed, R. (1999). *Reversal of virus-induced systemic shock and respiratory failure by blockade of the lymphotoxin pathway*. Nat Med ***5***, 1370-1374. 10.1038/70938.

30. Spahn, T.W., Maaser, C., Eckmann, L., Heidemann, J., Lugering, A., Newberry, R., Domschke, W., Herbst, H., and Kucharzik, T. (2004). *The lymphotoxin-beta receptor is critical for control of murine Citrobacter rodentium-induced colitis*. Gastroenterology ***127***, 1463-1473. 10.1053/j.gastro.2004.08.022.

31. Boehm, T., Scheu, S., Pfeffer, K., and Bleul, C.C. (2003). *Thymic medullary epithelial cell differentiation, thymocyte emigration, and the control of autoimmunity require lympho-epithelial cross talk via LTbetaR*. J Exp Med ***198***, 757-769. 10.1084/jem.20030794.

32. Cosway, E.J., Lucas, B., James, K.D., Parnell, S.M., Carvalho-Gaspar, M., White, A.J., Tumanov, A.V., Jenkinson, W.E., and Anderson, G. (2017). *Redefining thymus medulla specialization for central tolerance*. J Exp Med ***214***, 3183-3195. 10.1084/jem.20171000.

33. Seleznik, G., Seeger, H., Bauer, J., Fu, K., Czerkowicz, J., Papandile, A., Poreci, U., Rabah, D., Ranger, A., Cohen, C.D., Lindenmeyer, M., Chen, J., Edenhofer, I., Anders, H.J., Lech, M., Wuthrich, R.P., Ruddle, N.H., Moeller, M.J., Kozakowski, N., Regele, H., Browning, J.L., Heikenwalder, M., and Segerer, S. (2016). *The lymphotoxin beta receptor is a potential therapeutic target in renal inflammation*. Kidney Int ***89***, 113-126. 10.1038/ki.2015.280.

34. Zhang, G., Feizi, N., Zhao, D., Halesha, L., Williams, A.L., Randhawa, P.S., Abou-Daya, K.I., and Oberbarnscheidt, M.H. (2024). *Lymphotoxin beta receptor and tertiary lymphoid organs shape acute and chronic allograft rejection*. JCI Insight. 10.1172/jci.insight.177555.

35. Liu, H., Dai, H., Qiu, F., Chen, Y., Liang, C.L., Yang, B., Gong, N., Bromberg, J.S., and Dai, Z. (2024). *Electrostimulation suppresses allograft rejection via promoting lymphatic regulatory T cell migration mediated by lymphotoxin (LT)-LTbeta receptor signaling*. Am J Transplant. 10.1016/j.ajt.2024.06.019.

36. Fernandes, M.T., Dejardin, E., and dos Santos, N.R. (2016). *Context-dependent roles for lymphotoxin-beta receptor signaling in cancer development*. Biochim Biophys Acta ***1865***, 204-219. 10.1016/j.bbcan.2016.02.005.

37. Wolf, M.J., Seleznik, G.M., Zeller, N., and Heikenwalder, M. (2010). *The unexpected role of lymphotoxin beta receptor signaling in carcinogenesis: from lymphoid tissue formation to liver and prostate cancer development*. Oncogene ***29***, 5006-5018. 10.1038/onc.2010.260.

38. Dubey, J.P., Cerqueira-Cezar, C.K., Murata, F.H.A., Verma, S.K., Kwok, O.C.H., Pedersen, K., Rosenthal, B.M., and Su, C. (2020). *White-tailed deer (Odocoileus virginianus) are a reservoir of a diversity of Toxoplasma gondii strains in the USA and pose a risk to consumers of undercooked venison*. Parasitology ***147***, 775-781. 10.1017/S0031182020000451.

39. Zhang, Y., Li, D., Lu, S., and Zheng, B. (2022). *Toxoplasmosis vaccines: what we have and where to go?* NPJ Vaccines ***7***, 131. 10.1038/s41541-022-00563-0.

40. English, E.D., and Striepen, B. (2019). *The cat is out of the bag: How parasites know their hosts*. PLoS Biol ***17***, e3000446. 10.1371/journal.pbio.3000446.

41. Daher, D., Shaghlil, A., Sobh, E., Hamie, M., Hassan, M.E., Moumneh, M.B., Itani, S., El Hajj, R., Tawk, L., El Sabban, M., and El Hajj, H. (2021). *Comprehensive Overview of Toxoplasma gondii-Induced and Associated Diseases*. Pathogens ***10***. 10.3390/pathogens10111351.

42. Clough, B., and Frickel, E.M. (2017). *The Toxoplasma Parasitophorous Vacuole: An Evolving Host-Parasite Frontier*. Trends Parasitol ***33***, 473-488. 10.1016/j.pt.2017.02.007.

43. Melchor, S.J., and Ewald, S.E. (2019). *Disease Tolerance in Toxoplasma Infection*. Front Cell Infect Microbiol ***9***, 185. 10.3389/fcimb.2019.00185.

44. Khan, I.A., and Moretto, M. (2022). *Immune responses to Toxoplasma gondii*. Curr Opin Immunol ***77***, 102226. 10.1016/j.coi.2022.102226.

45. Iwasaki, A., and Medzhitov, R. (2010). *Regulation of adaptive immunity by the innate immune system*. Science ***327***, 291-295. 10.1126/science.1183021.

46. Sasai, M., and Yamamoto, M. (2019). *Innate, adaptive, and cell-autonomous immunity against Toxoplasma gondii infection*. Exp Mol Med ***51***, 1-10. 10.1038/s12276-019-0353-9.

47. Yarovinsky, F. (2014). *Innate immunity to Toxoplasma gondii infection*. Nat Rev Immunol ***14***, 109-121. 10.1038/nri3598.

48. Debierre-Grockiego, F., Campos, M.A., Azzouz, N., Schmidt, J., Bieker, U., Resende, M.G., Mansur, D.S., Weingart, R., Schmidt, R.R., Golenbock, D.T., Gazzinelli, R.T., and Schwarz, R.T. (2007). *Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived from Toxoplasma gondii*. J Immunol ***179***, 1129-1137. 10.4049/jimmunol.179.2.1129.

49. Koblansky, A.A., Jankovic, D., Oh, H., Hieny, S., Sungnak, W., Mathur, R., Hayden, M.S., Akira, S., Sher, A., and Ghosh, S. (2013). *Recognition of profilin by Toll-like receptor 12 is critical for host resistance to Toxoplasma gondii*. Immunity ***38***, 119-130. 10.1016/j.immuni.2012.09.016.

50. Plattner, F., Yarovinsky, F., Romero, S., Didry, D., Carlier, M.F., Sher, A., and Soldati-Favre, D. (2008). *Toxoplasma profilin is essential for host cell invasion and TLR11-dependent induction of an interleukin-12 response*. Cell Host Microbe ***3***, 77-87. 10.1016/j.chom.2008.01.001.

51. Yarovinsky, F., Zhang, D., Andersen, J.F., Bannenberg, G.L., Serhan, C.N., Hayden, M.S., Hieny, S., Sutterwala, F.S., Flavell, R.A., Ghosh, S., and Sher, A. (2005). *TLR11 activation of dendritic cells by a protozoan profilin-like protein*. Science ***308***, 1626-1629. 10.1126/science.1109893.

52. Aliberti, J., Reis e Sousa, C., Schito, M., Hieny, S., Wells, T., Huffnagle, G.B., and Sher, A. (2000). *CCR5 provides a signal for microbial induced production of IL-12 by CD8 alpha+ dendritic cells*. Nat Immunol ***1***, 83-87. 10.1038/76957.

53. Nishimura, M., Umeda, K., Suwa, M., Furuoka, H., and Nishikawa, Y. (2017). *CCR5 Is Involved in Interruption of Pregnancy in Mice Infected with Toxoplasma gondii during Early Pregnancy*. Infect Immun ***85***. 10.1128/IAI.00257-17.

54. Gazzinelli, R.T., Hieny, S., Wynn, T.A., Wolf, S., and Sher, A. (1993). *Interleukin 12 is required for the T-lymphocyte-independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts*. Proc Natl Acad Sci U S A ***90***, 6115-6119. 10.1073/pnas.90.13.6115.

55. Hunter, C.A., Subauste, C.S., Van Cleave, V.H., and Remington, J.S. (1994). *Production of gamma interferon by natural killer cells from Toxoplasma gondii-infected SCID mice: regulation by interleukin-10, interleukin-12, and tumor necrosis factor alpha*. Infect Immun ***62***, 2818-2824. 10.1128/iai.62.7.2818-2824.1994.

56. Lopez-Yglesias, A.H., Burger, E., Camanzo, E., Martin, A.T., Araujo, A.M., Kwok, S.F., and Yarovinsky, F. (2021). *T-bet-dependent ILC1- and NK cell-derived IFN-gamma mediates cDC1-dependent host resistance against Toxoplasma gondii*. PLoS Pathog ***17***, e1008299. 10.1371/journal.ppat.1008299.

57. Degrandi, D., Kravets, E., Konermann, C., Beuter-Gunia, C., Klumpers, V., Lahme, S., Rasch, E., Mausberg, A.K., Beer-Hammer, S., and Pfeffer, K. (2013). *Murine guanylate binding protein 2 (mGBP2) controls Toxoplasma gondii replication*. Proc Natl Acad Sci U S A ***110***, 294-299. 10.1073/pnas.1205635110.

58. Kravets, E., Degrandi, D., Weidtkamp-Peters, S., Ries, B., Konermann, C., Felekyan, S., Dargazanli, J.M., Praefcke, G.J., Seidel, C.A., Schmitt, L., Smits, S.H., and Pfeffer, K. (2012). *The GTPase activity of murine guanylate-binding protein 2 (mGBP2) controls the intracellular*

localization and recruitment to the parasitophorous vacuole of Toxoplasma gondii. J Biol Chem ***287***, 27452-27466. 10.1074/jbc.M112.379636.

59. Selleck, E.M., Fentress, S.J., Beatty, W.L., Degrandi, D., Pfeffer, K., Virgin, H.W.t., Macmicking, J.D., and Sibley, L.D. (2013). *Guanylate-binding protein 1 (Gbp1) contributes to cell-autonomous immunity against Toxoplasma gondii*. PLoS Pathog ***9***, e1003320. 10.1371/journal.ppat.1003320.

60. Steffens, N., Beuter-Gunia, C., Kravets, E., Reich, A., Legewie, L., Pfeffer, K., and Degrandi, D. (2020). *Essential Role of mGBP7 for Survival of Toxoplasma gondii Infection*. mBio ***11***. 10.1128/mBio.02993-19.

61. Yamamoto, M., Okuyama, M., Ma, J.S., Kimura, T., Kamiyama, N., Saiga, H., Ohshima, J., Sasai, M., Kayama, H., Okamoto, T., Huang, D.C., Soldati-Favre, D., Horie, K., Takeda, J., and Takeda, K. (2012). *A cluster of interferon-gamma-inducible p65 GTPases plays a critical role in host defense against Toxoplasma gondii*. Immunity ***37***, 302-313. 10.1016/j.immuni.2012.06.009.

62. Wilson, D.C., Matthews, S., and Yap, G.S. (2008). *IL-12 signaling drives CD8+ T cell IFN-gamma production and differentiation of KLRG1+ effector subpopulations during Toxoplasma gondii Infection*. J Immunol ***180***, 5935-5945. 10.4049/jimmunol.180.9.5935.

63. Yap, G., Pesin, M., and Sher, A. (2000). *Cutting edge: IL-12 is required for the maintenance of IFN-gamma production in T cells mediating chronic resistance to the intracellular pathogen, Toxoplasma gondii*. J Immunol ***165***, 628-631. 10.4049/jimmunol.165.2.628.

64. Sturge, C.R., Benson, A., Raetz, M., Wilhelm, C.L., Mirpuri, J., Vitetta, E.S., and Yarovinsky, F. (2013). *TLR-independent neutrophil-derived IFN-gamma is important for host resistance to intracellular pathogens*. Proc Natl Acad Sci U S A ***110***, 10711-10716. 10.1073/pnas.1307868110.

65. Casadevall, A. (2003). *Antibody-mediated immunity against intracellular pathogens: two-dimensional thinking comes full circle*. Infect Immun ***71***, 4225-4228. 10.1128/IAI.71.8.4225-4228.2003.

66. Kang, H., Remington, J.S., and Suzuki, Y. (2000). *Decreased resistance of B cell-deficient mice to infection with Toxoplasma gondii despite unimpaired expression of IFN-gamma, TNF-alpha, and inducible nitric oxide synthase*. J Immunol ***164***, 2629-2634. 10.4049/jimmunol.164.5.2629.

67. Sayles, P.C., Gibson, G.W., and Johnson, L.L. (2000). *B cells are essential for vaccination-induced resistance to virulent Toxoplasma gondii*. Infect Immun ***68***, 1026-1033. 10.1128/IAI.68.3.1026-1033.2000.

68. Couper, K.N., Roberts, C.W., Brombacher, F., Alexander, J., and Johnson, L.L. (2005). *Toxoplasma gondii-specific immunoglobulin M limits parasite dissemination by preventing host cell invasion*. Infect Immun ***73***, 8060-8068. 10.1128/IAI.73.12.8060-8068.2005.

69. Hammouda, N.A., Abo el-Naga, I., Hussein, E.D., and Rashwan, E.A. (1995). *Opsonization and intracellular killing of Toxoplasma gondii by human mononuclear phagocytes*. J Egypt Soc Parasitol ***25***, 11-17.

70. Johnson, L.L., Lanthier, P., Hoffman, J., and Chen, W. (2004). *Vaccination protects B cell-deficient mice against an oral challenge with mildly virulent Toxoplasma gondii*. Vaccine ***22***, 4054-4061. 10.1016/j.vaccine.2004.03.056.

71. Joiner, K.A., Fuhrman, S.A., Miettinen, H.M., Kasper, L.H., and Mellman, I. (1990). *Toxoplasma gondii: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts*. Science ***249***, 641-646. 10.1126/science.2200126.

72. Mineo, J.R., McLeod, R., Mack, D., Smith, J., Khan, I.A., Ely, K.H., and Kasper, L.H. (1993). *Antibodies to Toxoplasma gondii major surface protein (SAG-1, P30) inhibit infection of host cells and are produced in murine intestine after peroral infection*. J Immunol ***150***, 3951-3964.

73. Vercammen, M., Scorza, T., El Bouhdidi, A., Van Beeck, K., Carlier, Y., Dubremetz, J.F., and Verschueren, H. (1999). *Opsonization of Toxoplasma gondii tachyzoites with nonspecific immunoglobulins promotes their phagocytosis by macrophages and inhibits their proliferation in nonphagocytic cells in tissue culture*. Parasite Immunol ***21***, 555-563. 10.1046/j.1365-3024.1999.00256.x.

74. Glatman Zaretsky, A., Silver, J.S., Siwicki, M., Durham, A., Ware, C.F., and Hunter, C.A. (2012). *Infection with Toxoplasma gondii alters lymphotoxin expression associated with changes in splenic architecture*. Infect Immun ***80***, 3602-3610. 10.1128/IAI.00333-12.

75. Johnson, L.L., and Sayles, P.C. (2002). *Deficient humoral responses underlie susceptibility to Toxoplasma gondii in CD4-deficient mice*. Infect Immun ***70***, 185-191. 10.1128/IAI.70.1.185-191.2002.

76. Holodick, N.E., Rodriguez-Zhurbenko, N., and Hernandez, A.M. (2017). *Defining Natural Antibodies*. Front Immunol ***8***, 872. 10.3389/fimmu.2017.00872.

77. Bockstal, V., Geurts, N., and Magez, S. (2011). *Acute Disruption of Bone Marrow B Lymphopoiesis and Apoptosis of Transitional and Marginal Zone B Cells in the Spleen following a Blood-Stage Plasmodium chabaudi Infection in Mice*. J Parasitol Res ***2011***, 534697. 10.1155/2011/534697.

78. Bockstal, V., Guirnalda, P., Caljon, G., Goenka, R., Telfer, J.C., Frenkel, D., Radwanska, M., Magez, S., and Black, S.J. (2011). *T. brucei infection reduces B lymphopoiesis in bone marrow and truncates compensatory splenic lymphopoiesis through transitional B-cell apoptosis*. PLoS Pathog ***7***, e1002089. 10.1371/journal.ppat.1002089.

79. Amezcua Vesely, M.C., Bermejo, D.A., Montes, C.L., Acosta-Rodriguez, E.V., and Gruppi, A. (2012). *B-Cell Response during Protozoan Parasite Infections*. J Parasitol Res ***2012***, 362131. 10.1155/2012/362131.

80. Kobayashi, A., and Suzuki, Y. (1987). *Suppression of antibody responses by Toxoplasma infection in mice*. Zentralbl Bakteriol Mikrobiol Hyg A ***264***, 312-318. 10.1016/s0176-6724(87)80049-4.

81. Channon, J.Y., and Kasper, L.H. (1996). *Toxoplasma gondii-induced immune suppression by human peripheral blood monocytes: role of gamma interferon*. Infect Immun ***64***, 1181-1189. 10.1128/iai.64.4.1181-1189.1996.

82. Olias, P., Etheridge, R.D., Zhang, Y., Holtzman, M.J., and Sibley, L.D. (2016). *Toxoplasma Effector Recruits the Mi-2/NuRD Complex to Repress STAT1 Transcription and Block IFN-gamma-Dependent Gene Expression*. Cell Host Microbe ***20***, 72-82. 10.1016/j.chom.2016.06.006.

83. Matta, S.K., Olias, P., Huang, Z., Wang, Q., Park, E., Yokoyama, W.M., and Sibley, L.D. (2019). *Toxoplasma gondii effector TgIST blocks type I interferon signaling to promote infection*. Proc Natl Acad Sci U S A ***116***, 17480-17491. 10.1073/pnas.1904637116.

84. Pavia, C.S., Bittker, S.J., and Curnick, K.E. (1992). *Passive immunization protects guinea pigs from lethal Toxoplasma infection*. FEMS Microbiol Immunol ***4***, 97-104. 10.1111/j.1574-6968.1992.tb04975.x.

85. Cha, D.Y., Song, I.K., Lee, G.S., Hwang, O.S., Noh, H.J., Yeo, S.D., Shin, D.W., and Lee, Y.H. (2001). *Effects of specific monoclonal antibodies to dense granular proteins on the invasion of Toxoplasma gondii in vitro and in vivo*. Korean J Parasitol ***39***, 233-240. 10.3347/kjp.2001.39.3.233.

86. Kang, H.J., Kim, M.J., Chu, K.B., Lee, S.H., Moon, E.K., and Quan, F.S. (2021). *Passive Immunity and Antibody Response Induced by Toxoplasma gondii VLP Immunization*. Vaccines (Basel) ***9***. 10.3390/vaccines9050425.

87. Wu, M., Liu, S., Chen, Y., Liu, D., An, R., Cai, H., Wang, J., Zhou, N., Obed, C., Han, M., Shen, J., Chen, L., and Du, J. (2022). *Live-attenuated ME49Deltacdpk3 strain of Toxoplasma gondii protects against acute and chronic toxoplasmosis*. NPJ Vaccines ***7***, 98. 10.1038/s41541-022-00518-5.

88. Foster, B.G., and McCulloch, W.F. (1968). *Studies of active and passive immunity in animals inoculated with Toxoplasma gondii*. Can J Microbiol ***14***, 103-110. 10.1139/m68-018.

89. Strickland, G.T., and Sayles, P.C. (1977). *Depressed antibody responses to a thymus-dependent antigen in toxoplasmosis*. Infect Immun ***15***, 184-190. 10.1128/iai.15.1.184-190.1977.

90. Masihi, K.N., and Werner, H. (1977). *Suppression and enhancement of humoral immune response to Toxoplasma gondii by passive antibody*. Experientia ***33***, 1586-1587. 10.1007/BF01934012.

91. Wang, Y.G., Kim, K.D., Wang, J., Yu, P., and Fu, Y.X. (2005). *Stimulating lymphotoxin beta receptor on the dendritic cells is critical for their homeostasis and expansion*. J Immunol ***175***, 6997-7002. 10.4049/jimmunol.175.10.6997.

92. Suzuki, Y., Orellana, M.A., Schreiber, R.D., and Remington, J.S. (1988). *Interferon-gamma: the major mediator of resistance against Toxoplasma gondii*. Science ***240***, 516-518. 10.1126/science.3128869.

93. Askenase, M.H., Han, S.J., Byrd, A.L., Morais da Fonseca, D., Bouladoux, N., Wilhelm, C., Konkel, J.E., Hand, T.W., Lacerda-Queiroz, N., Su, X.Z., Trinchieri, G., Grainger, J.R., and Belkaid, Y. (2015). *Bone-Marrow-Resident NK Cells Prime Monocytes for Regulatory Function during Infection*. Immunity ***42***, 1130-1142. 10.1016/j.immuni.2015.05.011.

94. Wijdeven, R.H., van Luijn, M.M., Wierenga-Wolf, A.F., Akkermans, J.J., van den Elsen, P.J., Hintzen, R.Q., and Neefjes, J. (2018). *Chemical and genetic control of IFNgamma-induced MHCII expression*. EMBO Rep ***19***. 10.15252/embr.201745553.

95. Forthal, D.N. (2014). *Functions of Antibodies*. Microbiol Spectr ***2***, 1-17.

96. Winslow, G.M., Yager, E., Shilo, K., Volk, E., Reilly, A., and Chu, F.K. (2000). *Antibody-mediated elimination of the obligate intracellular bacterial pathogen Ehrlichia chaffeensis during active infection*. Infect Immun ***68***, 2187-2195. 10.1128/IAI.68.4.2187-2195.2000.

97. Joller, N., Weber, S.S., Muller, A.J., Sporri, R., Selchow, P., Sander, P., Hilbi, H., and Oxenius, A. (2010). *Antibodies protect against intracellular bacteria by Fc receptor-mediated lysosomal targeting*. Proc Natl Acad Sci U S A ***107***, 20441-20446. 10.1073/pnas.1013827107.

98. Shannon, J.G., Cockrell, D.C., Takahashi, K., Stahl, G.L., and Heinzen, R.A. (2009). *Antibody-mediated immunity to the obligate intracellular bacterial pathogen Coxiella burnetii is Fc receptor- and complement-independent*. BMC Immunol ***10***, 26. 10.1186/1471-2172-10-26.

99. Casadevall, A. (2018). *Antibody-based vaccine strategies against intracellular pathogens*. Curr Opin Immunol ***53***, 74-80. 10.1016/j.coi.2018.04.011.

100. Souza, S.P., Splitt, S.D., Sanchez-Arcila, J.C., Alvarez, J.A., Wilson, J.N., Wizzard, S., Luo, Z., Baumgarth, N., and Jensen, K.D.C. (2021). *Genetic mapping reveals Nfkbid as a central regulator of humoral immunity to Toxoplasma gondii*. PLoS Pathog ***17***, e1010081. 10.1371/journal.ppat.1010081.

101. C, S.S., and Hubal, A. (2023). *Animal Models for Toxoplasma gondii Infection*. Curr Protoc ***3***, e871. 10.1002/cpz1.871.

102. Watson, G.F., and Davis, P.H. (2019). *Systematic review and meta-analysis of variation in Toxoplasma gondii cyst burden in the murine model*. Exp Parasitol ***196***, 55-62. 10.1016/j.exppara.2018.12.003.

103. Luder, C.G., Algner, M., Lang, C., Bleicher, N., and Gross, U. (2003). *Reduced expression of the inducible nitric oxide synthase after infection with Toxoplasma gondii facilitates parasite replication in activated murine macrophages*. Int J Parasitol ***33***, 833-844. 10.1016/s0020-7519(03)00092-4.

104. Luder, C.G., Walter, W., Beuerle, B., Maeurer, M.J., and Gross, U. (2001). *Toxoplasma gondii down-regulates MHC class II gene expression and antigen presentation by murine macrophages via interference with nuclear translocation of STAT1alpha*. Eur J Immunol ***31***, 1475-1484. 10.1002/1521-4141(200105)31:5<1475::AID-IMMU1475>3.0.CO;2-C.

105. Zimmermann, S., Murray, P.J., Heeg, K., and Dalpke, A.H. (2006). *Induction of suppressor of cytokine signaling-1 by Toxoplasma gondii contributes to immune evasion in macrophages by blocking IFN-gamma signaling*. J Immunol ***176***, 1840-1847. 10.4049/jimmunol.176.3.1840.

106. Suzuki, Y., Joh, K., and Kobayashi, A. (1987). *Macrophage-mediated suppression of immune responses in Toxoplasma-infected mice. III. Suppression of antibody responses to parasite itself*. Cell Immunol ***110***, 218-225. 10.1016/0008-8749(87)90117-1.

107. Suzuki, Y., and Kobayashi, A. (1984). *Macrophage-mediated suppression of immune responses in Toxoplasma-infected mice. I. Inhibition of proliferation of lymphocytes in primary antibody responses*. Cell Immunol ***85***, 417-427. 10.1016/0008-8749(84)90255-7.

108. Suzuki, Y., and Kobayashi, A. (1985). *Macrophage-mediated suppression of immune responses in Toxoplasma-infected mice. II. Both H-2-linked and -nonlinked control of induction of suppressor macrophages*. Cell Immunol ***91***, 375-384. 10.1016/0008-8749(85)90235-7.

109. Suzuki, Y., Watanabe, N., and Kobayashi, A. (1981). *Nonspecific suppression of primary antibody responses and presence of plastic-adherent suppressor cells in Toxoplasma gondii-infected mice*. Infect Immun ***34***, 30-35. 10.1128/iai.34.1.30-35.1981.

110. Turner, P.V., Brabb, T., Pekow, C., and Vasbinder, M.A. (2011). *Administration of substances to laboratory animals: routes of administration and factors to consider*. J Am Assoc Lab Anim Sci ***50***, 600-613.

111. Taborda, C.P., Rivera, J., Zaragoza, O., and Casadevall, A. (2003). *More is not necessarily better: prozone-like effects in passive immunization with IgG*. J Immunol ***170***, 3621-3630. 10.4049/jimmunol.170.7.3621.

112. Goodner, K., and Horsfall, F.L. (1935). *The Protective Action of Type I Antipneumococcus Serum in Mice : I. The Quantitative Aspects of the Mouse Protection Test*. J Exp Med ***62***, 359-374. 10.1084/jem.62.3.359.

113. Heyman, B. (2000). *Regulation of antibody responses via antibodies, complement, and Fc receptors*. Annu Rev Immunol ***18***, 709-737. 10.1146/annurev.immunol.18.1.709.

114. Uhr, J.W., and Moller, G. (1968). *Regulatory effect of antibody on the immune response*. Adv Immunol ***8***, 81-127. 10.1016/s0065-2776(08)60465-4.

115. Xu, H., and Heyman, B. (2020). *IgG-mediated suppression of antibody responses: Hiding or snatching epitopes?* Scand J Immunol ***92***, e12921. 10.1111/sji.12921.

116. Joly, E., and Hudrisier, D. (2003). *What is trogocytosis and what is its purpose?* Nat Immunol ***4***, 815. 10.1038/ni0903-815.

117. Nakayama, Y., and Bromberg, J.S. (2012). *Lymphotoxin-beta receptor blockade induces inflammation and fibrosis in tolerized cardiac allografts*. Am J Transplant ***12***, 2322-2334. 10.1111/j.1600-6143.2012.04090.x.

118. Macho-Fernandez, E., Koroleva, E.P., Spencer, C.M., Tighe, M., Torrado, E., Cooper, A.M., Fu, Y.X., and Tumanov, A.V. (2015). *Lymphotoxin beta receptor signaling limits mucosal damage through driving IL-23 production by epithelial cells*. Mucosal Immunol ***8***, 403-413. 10.1038/mi.2014.78.

119. Shi, M., Deng, W., Bi, E., Mao, K., Ji, Y., Lin, G., Wu, X., Tao, Z., Li, Z., Cai, X., Sun, S., Xiang, C., and Sun, B. (2008). *TRIM30 alpha negatively regulates TLR-mediated NF-kappa B activation by targeting TAB2 and TAB3 for degradation*. Nat Immunol ***9***, 369-377. 10.1038/ni1577.

120. Wimmer, N., Heigl, U., Klingseisen, L., Schneider-Brachert, W., and Hehlgans, T. (2013). *Lymphotoxin-beta receptor signalling regulates cytokine expression via TRIM30alpha in a TRAF3-dependent manner*. Mol Immunol ***54***, 40-47. 10.1016/j.molimm.2012.10.036.

121. Jang, S.W., Lim, S.G., Suk, K., and Lee, W.H. (2015). *Activation of lymphotoxin-beta receptor enhances the LPS-induced expression of IL-8 through NF-kappaB and IRF-1*. Immunol Lett ***165***, 63-69. 10.1016/j.imlet.2015.04.001.

122. Mikami, Y., Matsuzaki, H., Horie, M., Noguchi, S., Jo, T., Narumoto, O., Kohyama, T., Takizawa, H., Nagase, T., and Yamauchi, Y. (2014). *Lymphotoxin beta receptor signaling induces IL-8 production in human bronchial epithelial cells*. PLoS One ***9***, e114791. 10.1371/journal.pone.0114791.

123. Stopfer, P., Mannel, D.N., and Hehlgans, T. (2004). *Lymphotoxin-beta receptor activation by activated T cells induces cytokine release from mouse bone marrow-derived mast cells*. J Immunol ***172***, 7459-7465. 10.4049/jimmunol.172.12.7459.

124. Wang, Y., Koroleva, E.P., Kruglov, A.A., Kuprash, D.V., Nedospasov, S.A., Fu, Y.X., and Tumanov, A.V. (2010). *Lymphotoxin beta receptor signaling in intestinal epithelial cells orchestrates innate immune responses against mucosal bacterial infection*. Immunity ***32***, 403-413. 10.1016/j.immuni.2010.02.011.

125. De Trez, C. (2012). *Lymphotoxin-beta receptor expression and its related signaling pathways govern dendritic cell homeostasis and function*. Immunobiology ***217***, 1250-1258. 10.1016/j.imbio.2012.06.010.

126. Mashayekhi, M., Sandau, M.M., Dunay, I.R., Frickel, E.M., Khan, A., Goldszmid, R.S., Sher, A., Ploegh, H.L., Murphy, T.L., Sibley, L.D., and Murphy, K.M. (2011). *CD8alpha(+) dendritic cells are the critical source of interleukin-12 that controls acute infection by Toxoplasma gondii tachyzoites*. Immunity ***35***, 249-259. 10.1016/j.immuni.2011.08.008.

127. Liu, C.H., Fan, Y.T., Dias, A., Esper, L., Corn, R.A., Bafica, A., Machado, F.S., and Aliberti, J. (2006). *Cutting edge: dendritic cells are essential for in vivo IL-12 production and development of resistance against Toxoplasma gondii infection in mice*. J Immunol ***177***, 31-35. 10.4049/jimmunol.177.1.31.

128. Sanecka, A., and Frickel, E.M. (2012). *Use and abuse of dendritic cells by Toxoplasma gondii*. Virulence ***3***, 678-689. 10.4161/viru.22833.

129. Courret, N., Darche, S., Sonigo, P., Milon, G., Buzoni-Gatel, D., and Tardieux, I. (2006). *CD11c- and CD11b-expressing mouse leukocytes transport single Toxoplasma gondii tachyzoites to the brain*. Blood ***107***, 309-316. 10.1182/blood-2005-02-0666.

130. Lambert, H., Vutova, P.P., Adams, W.C., Lore, K., and Barragan, A. (2009). *The Toxoplasma gondii-shuttling function of dendritic cells is linked to the parasite genotype*. Infect Immun ***77***, 1679-1688. 10.1128/IAI.01289-08.

131. Liesenfeld, O., Kang, H., Park, D., Nguyen, T.A., Parkhe, C.V., Watanabe, H., Abo, T., Sher, A., Remington, J.S., and Suzuki, Y. (1999). *TNF-alpha, nitric oxide and IFN-gamma are all critical for development of necrosis in the small intestine and early mortality in genetically susceptible*

mice infected perorally with Toxoplasma gondii. Parasite Immunol ***21***, 365-376. 10.1046/j.1365-3024.1999.00237.x.

132. Chen, J., Zhang, C., Yang, Z., Wu, W., Zou, W., Xin, Z., Zheng, S., Liu, R., Yang, L., and Peng, H. (2024). *Intestinal microbiota imbalance resulted by anti-Toxoplasma gondii immune responses aggravate gut and brain injury*. Parasit Vectors ***17***, 284. 10.1186/s13071-024-06349-8.

133. Hatter, J.A., Kouche, Y.M., Melchor, S.J., Ng, K., Bouley, D.M., Boothroyd, J.C., and Ewald, S.E. (2018). *Toxoplasma gondii infection triggers chronic cachexia and sustained commensal dysbiosis in mice*. PLoS One ***13***, e0204895. 10.1371/journal.pone.0204895.

134. Liesenfeld, O., Kosek, J., Remington, J.S., and Suzuki, Y. (1996). *Association of CD4+ T cell-dependent, interferon-gamma-mediated necrosis of the small intestine with genetic susceptibility of mice to peroral infection with Toxoplasma gondii*. J Exp Med ***184***, 597-607. 10.1084/jem.184.2.597.

135. Suzuki, Y., Sher, A., Yap, G., Park, D., Neyer, L.E., Liesenfeld, O., Fort, M., Kang, H., and Gufwoli, E. (2000). *IL-10 is required for prevention of necrosis in the small intestine and mortality in both genetically resistant BALB/c and susceptible C57BL/6 mice following peroral infection with Toxoplasma gondii*. J Immunol ***164***, 5375-5382. 10.4049/jimmunol.164.10.5375.

136. Walker, W., Roberts, C.W., Ferguson, D.J., Jebbari, H., and Alexander, J. (1997). *Innate immunity to Toxoplasma gondii is influenced by gender and is associated with differences in interleukin-12 and gamma interferon production*. Infect Immun ***65***, 1119-1121. 10.1128/IAI.65.3.1119-1121.1997.

137. Alonaizan, R., Woods, S., Hargrave, K.E., and Roberts, C.W. (2021). *An Exaggerated Immune Response in Female BALB/c Mice Controls Initial Toxoplasma gondii Multiplication but Increases Mortality and Morbidity Relative to Male Mice*. Pathogens ***10***. 10.3390/pathogens10091154.

138. Garfoot, A.L., Cervantes, P.W., and Knoll, L.J. (2019). *Transcriptional Analysis Shows a Robust Host Response to Toxoplasma gondii during Early and Late Chronic Infection in Both Male and Female Mice*. Infect Immun ***87***. 10.1128/IAI.00024-19.

139. Scotland, R.S., Stables, M.J., Madalli, S., Watson, P., and Gilroy, D.W. (2011). *Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female mice*. Blood ***118***, 5918-5927. 10.1182/blood-2011-03-340281.

140. Gay, L., Melenotte, C., Lakbar, I., Mezouar, S., Devaux, C., Raoult, D., Bendiane, M.K., Leone, M., and Mege, J.L. (2021). *Sexual Dimorphism and Gender in Infectious Diseases*. Front Immunol ***12***, 698121. 10.3389/fimmu.2021.698121.

141. Clausen, B.E., Burkhardt, C., Reith, W., Renkawitz, R., and Forster, I. (1999). *Conditional gene targeting in macrophages and granulocytes using LysMcre mice*. Transgenic Res ***8***, 265-277. 10.1023/a:1008942828960.

142. Stranges, P.B., Watson, J., Cooper, C.J., Choisy-Rossi, C.M., Stonebraker, A.C., Beighton, R.A., Hartig, H., Sundberg, J.P., Servick, S., Kaufmann, G., Fink, P.J., and Chervonsky, A.V. (2007). *Elimination of antigen-presenting cells and autoreactive T cells by Fas contributes to prevention of autoimmunity*. Immunity ***26***, 629-641. 10.1016/j.immuni.2007.03.016.

143. Ma, W., Tessarollo, L., Hong, S.B., Baba, M., Southon, E., Back, T.C., Spence, S., Lobe, C.G., Sharma, N., Maher, G.W., Pack, S., Vortmeyer, A.O., Guo, C., Zbar, B., and Schmidt, L.S. (2003). *Hepatic vascular tumors, angiectasis in multiple organs, and impaired spermatogenesis in mice with conditional inactivation of the VHL gene*. Cancer Res ***63***, 5320-5328.

3.3 Lymphotoxin β Receptor: a Crucial Role in Innate and Adaptive Immune Responses Against *Toxoplasma gondii*

Authors

Anne Tersteegen, Ursula R. Sorg, Richard Virgen-Slane, **Marcel Helle**, Patrick Petzsch, Karl Köhrer, Daniel Degrandi, Carl F. Ware, Klaus Pfeffer

Published in

Infection and Immunity (2021)

Impact factor

2.9 (2023)

Own contributions to this work

Overall: 5%

Conducted all of the following experiments:

qRT-PCRs, confocal microscopy

Other major contributions:

-

Lymphotoxin β receptor: a Crucial Role in Innate and Adaptive Immune Responses against *Toxoplasma gondii*

Anne Tersteegen[1], Ursula R. Sorg[1], Richard Virgen-Slane[2], **Marcel Helle**[1], Patrick Petzsch[3], Karl Köhrer[3], Daniel Degrandi[1], Carl F. Ware[2], Klaus Pfeffer[1]*

[1]Institute of Medical Microbiology and Hospital Hygiene, Heinrich Heine University, Düsseldorf, Germany.

[2]Laboratory of Molecular Immunology, Infectious and Inflammatory Diseases Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA 92037.

[3]Biological and Medical Research Center (BMFZ), Heinrich Heine University, Düsseldorf, Germany.

*: To whom correspondence should be addressed

Prof. Dr. Klaus Pfeffer

Institute of Medical Microbiology and Hospital Hygiene

Heinrich Heine University Düsseldorf

Universitätsstraße 1

40225 Düsseldorf, Germany

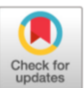
Phone: +49 (0) 211 81-12459

Fax: +49 (0) 211 81-15906

Email: klaus.pfeffer@hhu.de

Keywords: lymphotoxin beta receptor*, Toxoplasma gondii*, immunity, innate and adaptive immune response, infection, mouse

HOST RESPONSE AND INFLAMMATION



Lymphotoxin β Receptor: a Crucial Role in Innate and Adaptive Immune Responses against *Toxoplasma gondii*

Anne Tersteegen,[a,d] Ursula R. Sorg,[a] Richard Virgen-Slane,[b] Marcel Helle,[a] Patrick Petzsch,[c] Ildiko R. Dunay,[d] Karl Köhrer,[c] Daniel Degrandi,[a] Carl F. Ware,[b] Klaus Pfeffer[a]

[a]Institute of Medical Microbiology and Hospital Hygiene, Heinrich Heine University, Düsseldorf, Germany
[b]Laboratory of Molecular Immunology, Infectious and Inflammatory Diseases Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, California, USA
[c]Biological and Medical Research Center (BMFZ), Heinrich Heine University, Düsseldorf, Germany
[d]Institute of Inflammation and Neurodegeneration, Otto-von-Guericke-University, Magdeburg, Germany

ABSTRACT The lymphotoxin β receptor (LTβR) plays an essential role in the initiation of immune responses to intracellular pathogens. In mice, the LTβR is crucial for surviving acute toxoplasmosis; however, until now, a functional analysis was largely incomplete. Here, we demonstrate that the LTβR is a key regulator required for the intricate balance of adaptive immune responses. *Toxoplasma gondii*-infected LTβR-deficient (LTβR$^{-/-}$) mice show globally altered interferon-γ (IFN-γ) regulation, reduced IFN-γ-controlled host effector molecule expression, impaired T cell functionality, and an absent anti-parasite-specific IgG response, resulting in a severe loss of immune control of the parasites. Reconstitution of LTβR$^{-/-}$ mice with toxoplasma immune serum significantly prolongs survival following *T. gondii* infection. Notably, analysis of RNA-seq data clearly indicates a specific effect of *T. gondii* infection on the B cell response and isotype switching. This study uncovers the decisive role of the LTβR in cytokine regulation and adaptive immune responses to control *T. gondii*.

KEYWORDS lymphotoxin, *Toxoplasma gondii*, host-pathogen interactions

The lymphotoxin β receptor (LTβR) is one of the core members of the tumor necrosis factor (TNF)/TNF receptor (TNFR) superfamily (1, 2). It has two cognate ligands, LTβ (LT$\alpha_1\beta_2$) and LIGHT (homologous to lymphotoxins, exhibits inducible expression, and competes with herpesvirus [HSV] glycoprotein D for herpesvirus entry mediator [HVEM], a receptor expressed by T lymphocytes) (3, 4). LTβR-mediated signaling is known to be essential for the organogenesis of secondary lymphoid tissues, the maintenance of their structure, and its role in mediating innate immune responses to many pathogens is also well documented (2, 5–7). LTβR-deficient (LTβR$^{-/-}$) mice lack lymph nodes (LNs) and Peyer's patches (PPs), show reduced numbers of natural killer (NK) cells and dendritic cells (DCs) as well as impaired immunoglobulin (Ig) affinity maturation (7, 8). In infection models, LTβR$^{-/-}$ mice show pronounced defects in their immune response against *Listeria monocytogenes*, *Mycobacterium tuberculosis* (5), cytomegalovirus (9), lymphocytic choriomeningitis virus (LCMV) (10), and Zika virus (11), as well as *Toxoplasma gondii* (*T. gondii*) (12). In spite of these extensive deficits, not much is known about the exact role of LTβR signaling for efficient generation of the immune response against pathogens.

T. gondii, the causative agent of toxoplasmosis, is an obligate intracellular parasite belonging to the Apicomplexa. It is able to invade most warm-blooded vertebrates, including humans (13, 14), and can infect all nucleated cells. While acute toxoplasmosis usually presents with only mild, flu-like symptoms in immunocompetent hosts, it sometimes manifests as lymphadenitis, hepatosplenomegaly, myocarditis, or pneumonia. In immunocompromised patients, toxoplasmosis can cause serious health

Citation Tersteegen A, Sorg UR, Virgen-Slane R, Helle M, Petzsch P, Dunay IR, Köhrer K, Degrandi D, Ware CF, Pfeffer K. 2021. Lymphotoxin β receptor: a crucial role in innate and adaptive immune responses against *Toxoplasma gondii*. Infect Immun 89:e00026-21. https://doi.org/10.1128/IAI.00026-21.

Editor De'Broski R. Herbert, University of Pennsylvania

Copyright © 2021 Tersteegen et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Address correspondence to Klaus Pfeffer, klaus.pfeffer@hhu.de.

Received 15 January 2021
Returned for modification 25 February 2021
Accepted 12 March 2021
Accepted manuscript posted online 22 March 2021
Published 17 May 2021

problems and, when primary infection occurs during pregnancy, severe congenital defects may occur (15–17).

The early immune response to *T. gondii* is characterized by recognition of *T. gondii*-associated molecules (i.e., profilin) by different cell types, such as DCs. These cells produce distinct cytokines in response to infection, such as interleukin-12 (IL-12) and TNF, thus activating and stimulating other cell types, including NK cells (18), T cells (19), innate lymphoid cells (ILCs) (20), and macrophages (21), which in turn, produce inflammatory cytokines such as IFN-γ.

IFN-γ signaling is essential for limiting *T. gondii* proliferation during the acute stage of toxoplasmosis and driving the parasite into the chronic stage, where it is contained by a functional immune response (22–25). IFN-γ-driven effector mechanisms include induction of cell-autonomous effector mechanisms (26, 27), such as depletion of tryptophan (28) and reactive nitrogen production (29), which suppress *T. gondii* replication and are essential for restricting parasite growth. IFN-γ also strongly induces murine guanylate-binding proteins (mGBPs), which play a major role in restricting parasite growth of *T. gondii* as well as other intracellular pathogens (30–33). Within an infected cell, *T. gondii* resides within a parasitophorous vacuole (PV) that effectively protects the parasite from lysosomal activity (34). mGBPs are recruited to the PV and are instrumental in destroying first the PV and then the parasites within (30, 31, 33, 35, 36).

Previous studies have shown that other core members of the TNF/TNFR superfamily, such as the ligands TNF and LTα, which signal via the TNFRI receptor, also play an important part in the immune response to *T. gondii* (25, 37, 38). However, there is only limited data published on the role of the LTβR; it has been demonstrated that signaling via the LTβR is essential for the upregulation of mGBPs after *T. gondii* infection as well as for overall survival (12). Glatman Zaretsky et al. have shown that LTβ signaling is important for maintaining intact splenic architecture and, indirectly, for efficient *T. gondii*-specific antibody production in *T. gondii* type II strains (Prugniaud) (39). Nevertheless, the pathophysiology responsible for the increased susceptibility of LTβR$^{-/-}$ mice to *T. gondii* infection is still elusive.

Here, we demonstrate that LTβR deficiency results in dramatically dysregulated IFN-γ responses, impaired expression of antiparasite effector molecules, limited T cell functionality, and an abrogated *T. gondii* specific IgG response. We show that by transfer of *T. gondii* immune serum, survival of LTβR$^{-/-}$ mice can be prolonged, demonstrating that the susceptibility of LTβR$^{-/-}$ mice to *T. gondii* infection is possibly due to a direct role of LTβR signaling in Ig class switch. These results lead to a new understanding of LTβR-mediated immunity and the pathophysiology of toxoplasmosis and will hopefully aid in developing much-needed new treatment and prevention options such as passive vaccination strategies for human toxoplasmosis.

RESULTS

LTβR deficiency leads to increased parasite burden in lung, spleen, and muscle. While wild-type C57BL/6 (WT) mice survive a *T. gondii* infection, LTβR$^{-/-}$ mice are highly susceptible to *T. gondii* infection and do not survive beyond day 14 postinfection (p.i.) (Fig. 1a). This high susceptibility is in accordance with our previous study (12). To characterize the cause of this susceptibility in LTβR$^{-/-}$ mice, we first assessed the parasite burden in *T. gondii*-infected WT and LTβR$^{-/-}$ animals during the acute phase of infection via quantitative realtime PCR (qRT-PCR) (Fig. 1b). In lung tissue, we found increasing amounts of *T. gondii* DNA up to day 10 p.i. in both cohorts, with significantly larger amounts in LTβR$^{-/-}$ than WT mice on day 10 p.i. In the spleen, *T. gondii* DNA amounts increased only moderately in WT mice through the course of infection (Fig. 1b). In contrast, LTβR$^{-/-}$ mice showed a significant increase of *T. gondii* DNA by day 10 p.i. and significantly increased amounts compared to WT mice on days 7 and 10 p.i. Interestingly, in both genotypes, reduced amounts of *T. gondii* DNA were detected on day 10 compared to day 7 p.i. Similar results were observed in muscle tissue (Fig. 1b). In WT mice, the parasite burden rose only moderately, while LTβR$^{-/-}$ mice showed a significant increase by day 10 p.i. as well as significantly larger amounts on days 7 and 10 p.i. To summarize, LTβR$^{-/-}$ mice showed increased parasite

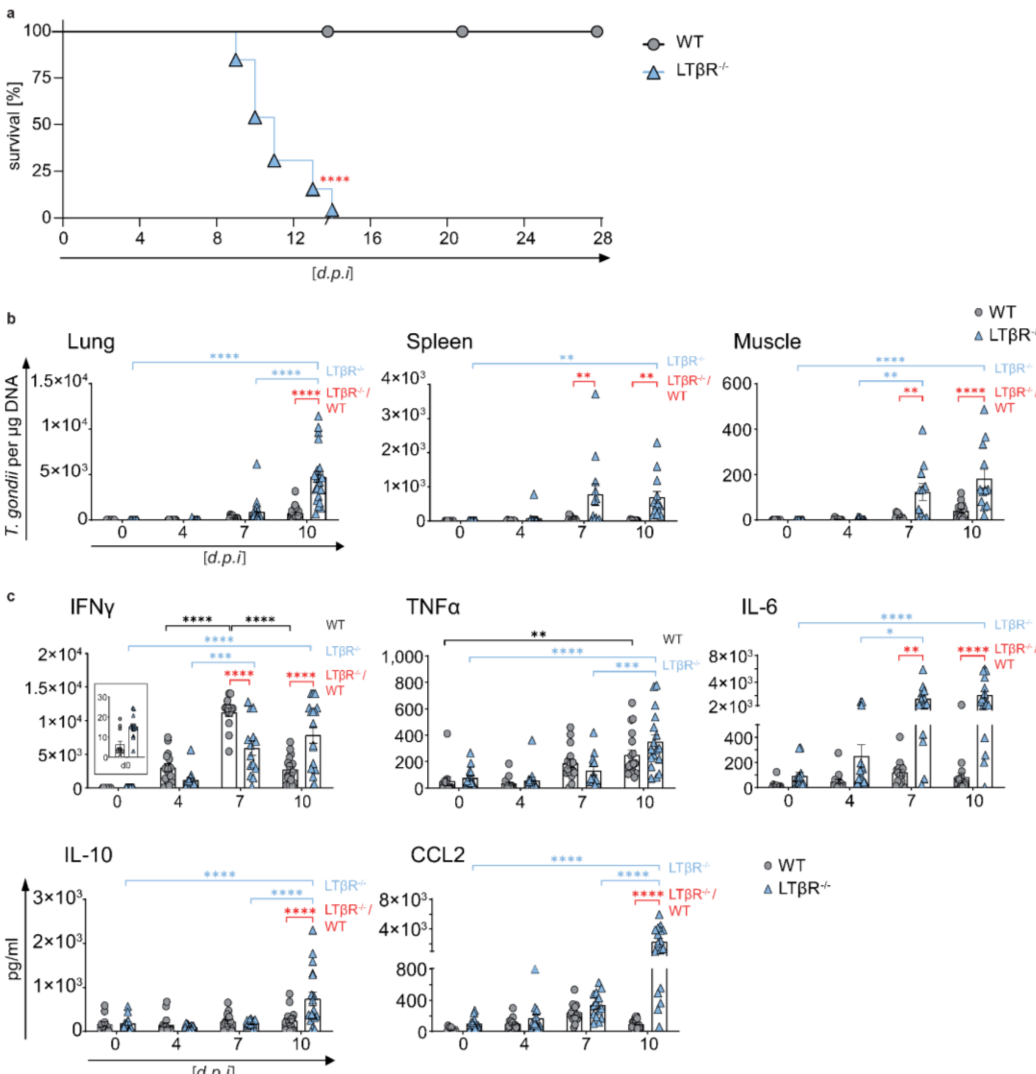


FIG 1 LTβR$^{-/-}$ mice show increased parasite load and dysregulated cytokine expression. (a) Survival of *T. gondii*-infected (ME49, 40 cysts, i.p.) WT ($n = 15$) and LTβR$^{-/-}$ ($n = 13$) mice. (b) qRT-PCR analysis of *T. gondii* DNA (assessing parasite load) in lung, spleen, and muscle tissue of uninfected (d0) and *T. gondii*-infected WT and LTβR$^{-/-}$ mice (d0 to d7, $n \geq 12$; d10, $n \geq 14$). (c) Expression of IFN-γ, TNF-α, IL-6, IL-10, and CCL2 in the serum of uninfected and *T. gondii*-infected WT and LTβR$^{-/-}$ mice (d0 to d7, $n \geq 12$; d10, $n = 18$) analyzed via bead-based immunoassay. The data shown represent at least three independent experiments; symbols represent individual animals, columns represent mean values, and error bars represent the ± SEM. A log rank (Mantel Cox) test was used for statistical analysis represented in panel a. Two-way ANOVA corrected for multiple comparison using Tukey's *post hoc* test was used for the statistical analysis represented in panels b and c. *, $P < 0.0332$; **, $P < 0.0021$; ***, $P < 0.0002$; ****, $P < 0.0001$.

burden compared to WT mice, pointing toward a failure of these animals to adequately control parasite proliferation in the acute phase of infection.

Dysregulated cytokines in the serum of LTβR$^{-/-}$ mice after infection with *T. gondii*. Since cytokines, especially IFN-γ and TNF-α, as signature molecules of a Th1 response play an important role in containing *T. gondii* expansion (16, 22, 40), we analyzed cytokine amounts in sera of infected mice (Fig. 1c). In both genotypes IFN-γ amounts increased slightly by day 4 p.i. In WT animals, IFN-γ amounts increased significantly by day 7 p.i. but were found to be markedly decreased again on day 10 p.i. While LTβR$^{-/-}$ mice also showed a significant increase of IFN-γ expression on day 7 p.i., amounts were

significantly lower than those of WT animals. Also, in LTβR$^{-/-}$ mice, IFN-γ expression levels were significantly higher on day 10 p.i. than those of WT animals. TNF-α expression increased significantly in WT as well as LTβR$^{-/-}$ animals by day 10 p.i. and did not differ significantly between the two genotypes, although amounts in LTβR$^{-/-}$ mice seemed to rise more steeply later in infection (day 7 versus day 10 p.i. for WT and LTβR$^{-/-}$ mice, respectively).

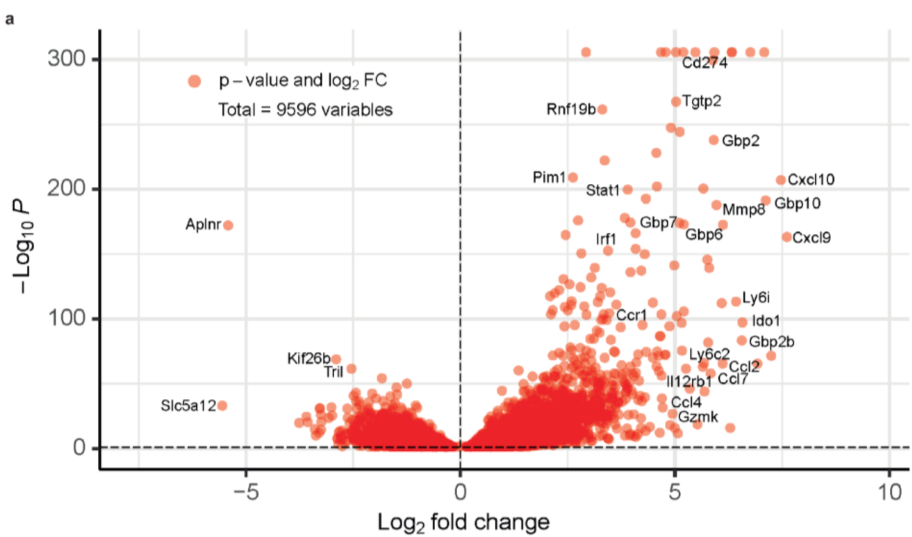
In WT animals, expression of IL-6, another proinflammatory cytokine (41), was slightly increased on day 4 and day 7 p.i. but was reduced again on day 10 p.i. (Fig. 1c). In contrast, in LTβR$^{-/-}$ mice, IL-6 amounts rose significantly during the course of infection and were significantly higher on days 7 and 10 p.i. than those of WT mice. Amounts of IL-10, known for its anti-inflammatory properties during infection (42), did not change significantly in WT animals during the course of infection (Fig. 1c). In contrast, amounts in LTβR$^{-/-}$ animals rose significantly on day 10 p.i. and were significantly higher than those of WT mice. The monocyte chemotactic factor (CCL2), a chemokine described to be induced by *T. gondii* (43), increased in WT as well as LTβR$^{-/-}$ mice on days 4 and 7 p.i. But while CCL2 in WT mice declined again by day 10 p.i., CCL2 further increased in LTβR$^{-/-}$ mice on day 10 p.i. and was significantly higher than in WT mice (Fig. 1c). Interestingly, LTβR$^{-/-}$ mice showed increased baseline amounts (day 0) for IFN-γ, TNF-α, IL-6, and CCL2 compared to WT mice, even though these differences were not significant.

Significantly different amounts were detected for IFN-β, IL-1α, IL-23, and IL-27 only on day 4 p.i. (see Fig. S1 in the supplemental material). LTβR$^{-/-}$ animals showed increased baseline amounts (day 0) for IFN-β, IL-1α, IL-1β, IL-17A, IL-23, IL-27, and IL-12p70, which were, however, significant only in the case of IL-1β. No differences in IL-12p70 levels were detected for the two genotypes (Fig. S1).

To summarize, uninfected LTβR$^{-/-}$ mice show different baseline amounts of proinflammatory cytokines, suggesting a subtle activation of the immune system. Furthermore, in these animals, the coordinated immune defense during *T. gondii* infection is dysregulated.

Markedly altered transcriptome in the lungs of LTβR$^{-/-}$ mice after *T. gondii* infection. The lungs are one of the target organs of *T. gondii* tachyzoite dissemination (12, 44). In line with that observation, we detected large amounts of *T. gondii* DNA in lung tissue of LTβR$^{-/-}$ compared to WT mice on day 4 p.i. (Fig. 1b). To determine whether WT and LTβR$^{-/-}$ mice show differences in global gene expression patterns in the lungs, we analyzed lung tissue via transcriptome sequencing (RNA-seq) on day 7 p.i. Interestingly, gene set enrichment analysis (GSEA) of these data showed a significant upregulation of Gene Ontology (GO) (biological process) molecular signatures for "response to type I interferons, response to interferon gamma, and interferon gamma mediated signaling pathway" in *T. gondii*-infected WT compared to LTβR$^{-/-}$ mice on day 7 p.i. (Fig. S2). The data depicted by a volcano plot (Fig. 2a) clearly show a significant upregulation of IFN-γ-regulated genes in *T. gondii*-infected WT mice compared to LTβR$^{-/-}$ mice (day 7 p.i.); for instance, transcripts for mGBPs (mGBP2b/1, 2, 6, 7, and 10), transcripts for effector molecules (IDO1, Gzmk), transcripts for chemokines and chemokine receptors responsible for recruitment of immune cells (CCL2, CCL4, CCL7, CXCL9, CXCL10, CCR1), transcripts for proteins involved in IFN-γ signaling (IRF1, STAT1), transcripts induced by IFN-γ (TGTP1, PIM1), and other transcripts known to be involved in immune responses (CD274, IL12Rb1, Ly6i, Ly6c2, MMP8, RNF19b) were found to be highly expressed in infected WT but not in LTβR$^{-/-}$ lungs on day 7 p.i. This suggests that LTβR$^{-/-}$ mice fail to adequately upregulate (IFN-γ-dependent) immune responses in the lung.

LTβR deficiency leads to dysregulation of cytokine expression in the lung. To add additional kinetic data RNA-seq data (Fig. 2a) and cytokine levels in serum (Fig. 1c; Fig. S1), we determined mRNA expression levels of cytokines in the lungs of infected WT and LTβR$^{-/-}$ mice at several time points after infection (Fig. 2b and c). Baseline expression levels of IFN-γ were higher in LTβR$^{-/-}$ animals; thus, while levels rose on day 4 p.i. in both genotypes, this increase was only significant in WT mice (Fig. 2b). While IFN-γ mRNA levels were markedly decreased in WT mice by day 10 p.i., they were still markedly but not significantly elevated in LTβR$^{-/-}$ mice (Fig. 2b). Baseline expression of TNF-α was



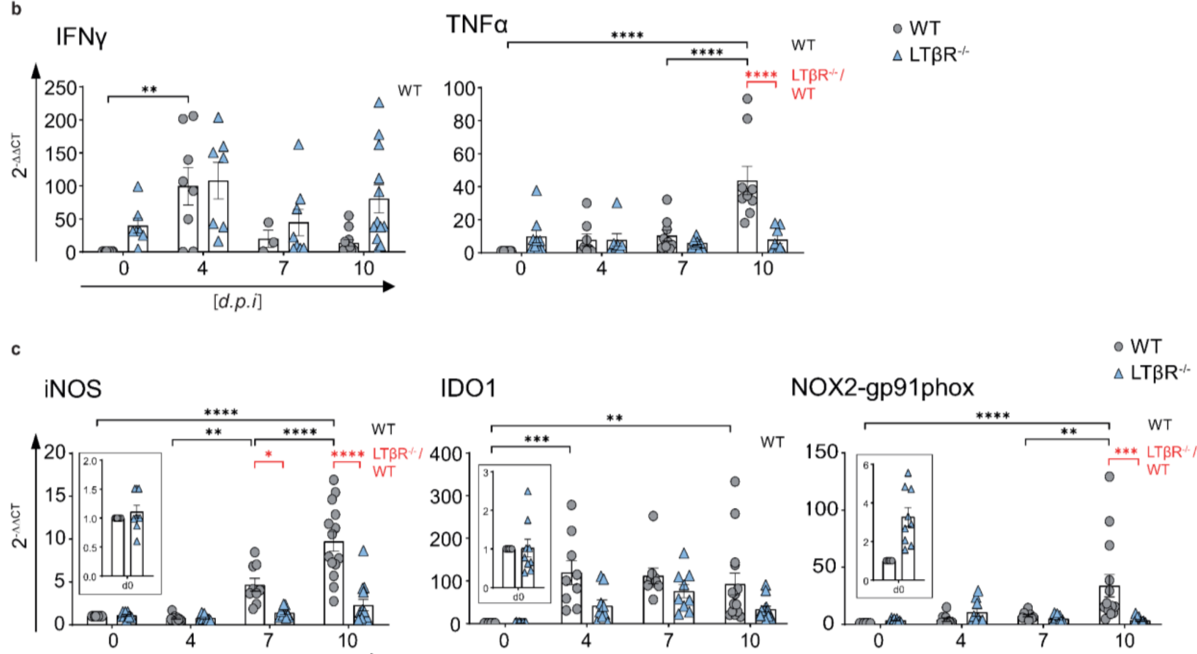


FIG 2 Lungs of LTβR$^{-/-}$ mice show an altered transcriptome after *T. gondii* infection. (a) Volcano plot showing RNA-seq data of lung tissue of infected WT mice correlated with infected LTβR$^{-/-}$ mice (d7 p.i.; $n = 3$/group). The dashed horizontal black line represents an adjusted P value of 0.1 (Wald test). (b and c) qRT-PCR analysis of (b) cytokines (IFN-γ and TNF-α) and (c) host effector molecules (iNOS, IDO1, NOX2-gp91phox) in lung tissue from uninfected (d0) and *T. gondii*-infected (ME49, 40 cysts, i.p.) WT and LTβR$^{-/-}$ mice (d0 to 7, $n \geq 12$; d10, $n \geq 14$; exception: IFN-γ, $n \geq 3$, d0 to 10 p.i.). Data shown in panels b and c represent four independent experiments; symbols represent individual animals, columns represent mean values, and error bars represent the ± SEM. Two-way ANOVA corrected for multiple comparison using Tukey's *post hoc* test was used for the statistical analysis. *, $P < 0.0332$; **, $P < 0.0021$; ***, $P < 0.0002$; ****, $P < 0.0001$.

increased in LTβR$^{-/-}$ mice but did not change significantly during the course of infection. WT mice showed a significant increase in TNF-α expression on day 10 p.i. (Fig. 2b), indicating a significant difference in the cytokine response between the two genotypes on day 10 p.i. Baseline expression levels of LTβ were significantly increased in LTβR$^{-/-}$ mice, which could

be due to a lack of negative feedback or compensatory mechanisms. However, while levels tended to be higher in LTβR$^{-/-}$ animals throughout the infection, there were no significant differences in LTβ expression between the two genotypes (Fig. S3). IL-4 expression was significantly increased in WT animals on day 10 p.i. compared to baseline expression. In LTβR$^{-/-}$ mice, IL-4 expression was comparable to that of WT mice but not significantly increased on day 10 p.i. compared to baseline expression (Fig. S3). These data confirm that LTβR$^{-/-}$ mice show a dysregulated immune homeostasis not only in serum (Fig. 1c; Fig. S1) but also in lung tissue after *T. gondii* infection.

LTβR deficiency leads to impaired IFN-γ-regulated effector molecule expression in the lung. IFN-γ-regulated effector molecules are pivotal in *T. gondii* elimination (31, 33, 45), having important immune response functions. In particular, the roles of effector molecules, such as iNOS, IDO, and NOX2-gp91phox (46–48), are well documented. Since RNA-seq data (Fig. 2a) showed high expression of effector molecules in infected WT, but not LTβR$^{-/-}$ (31) mice we assessed the expression of major effector molecules in lungs by qRT-PCR next (Fig. 2c). In contrast to WT mice, LTβR$^{-/-}$ mice failed to upregulate iNOS expression postinfection, leading to significant differences between the two genotypes on days 7 and 10 p.i. WT mice showed significant upregulation of IDO1 expression on day 4 p.i. and had significantly increased IDO1 expression levels on day 10 p.i., whereas LTβR$^{-/-}$ mice showed only a minor increase of IDO1 expression, and this difference was not significant compared to baseline expression. NOX2-gp91phox presented a similar picture—significantly increased NOX2-gp91phox expression in WT animals on day 10 p.i. compared to baseline expression as well as compared to LTβR$^{-/-}$ mice and a complete failure of upregulation of NOX2-gp91phox in the absence of LTβR. The failure to adequately upregulate IFN-γ-regulated effector molecules involved in cell intrinsic defense mechanisms essential for suppressing *T. gondii* replication most likely contributes to the increased parasite burden observed in LTβR$^{-/-}$ animals.

LTβR deficiency leads to impaired IFN-γ-induced mGBP expression and IFN-γ signaling in the lung. Another important group of genes upregulated in an IFN-γ-dependent manner after *T. gondii* infection are mGBPs (30). These GTPases have been shown to be essentially involved in *T. gondii* elimination (30–33). A heat map for mGBP expression data (Fig. 3a) was generated from the RNA-seq data, illustrating an overall slight increase in baseline mGBP expression in uninfected (day 0) LTβR$^{-/-}$ mice compared to WT mice but an overall lower mGBP expression in LTβR$^{-/-}$ compared to WT mice on day 7 p.i. These results were confirmed by qRT-PCR analysis of mGBP mRNA expression; for all mGBPs analyzed (mGBP1, 2, 3, 5, 6/10, 7, 8, and 9), we observed a significant increase in mGBP expression ($P < 0.0001$ in all cases) in WT animals by day 10 p.i. (Fig. 3b). In contrast, in LTβR$^{-/-}$ mice, a significant rise on day 10 p.i. compared to baseline expression was only observed for mGBP2, mGBP3, and mGBP7. Also, expression levels of all mGBPs were significantly higher in WT mice than in to LTβR$^{-/-}$ mice on day 10 p.i., with the exception of mGBP6/10, where expression levels were only slightly increased in WT mice. The failure to adequately upregulate expression of mGBPs early after *T. gondii* infection was further confirmed by immunoblot analysis, where upregulation of mGBP2 and mGBP7 protein expression was already detectable on day 4 p.i. in WT mice but not in LTβR$^{-/-}$ mice (Fig. 3c; Fig. S4). This defect in upregulation of mGBP expression after *T. gondii* expression likely has a major effect on the ability of LTβR$^{-/-}$ mice to contain parasite replication, as mGBPs are essential for an effective immune response against this parasite (31–33).

Since protein expression of IFN-γ-induced mGBPs was affected in lungs of LTβR$^{-/-}$ mice in *T. gondii* infection, we further analyzed protein expression of prototype genes directly involved in IFN-γR signaling (Fig. 3c; Fig. S4). Protein expression levels of STAT1, pSTAT1, IRF-1, and pSTAT3 increased in WT mice during the course of infection. In contrast, LTβR$^{-/-}$ animals showed a marked delay in the upregulation of these proteins. In WT animals, JAK1 and STAT3 expression increased until day 7 p.i. but decreased again on day 10 p.i. In uninfected LTβR$^{-/-}$ mice, expression of these proteins was higher than in uninfected WT mice but did not increase early in infection. This also provides evidence for an altered IFN-γ/IFN-γR signaling axis during *T. gondii* infection.

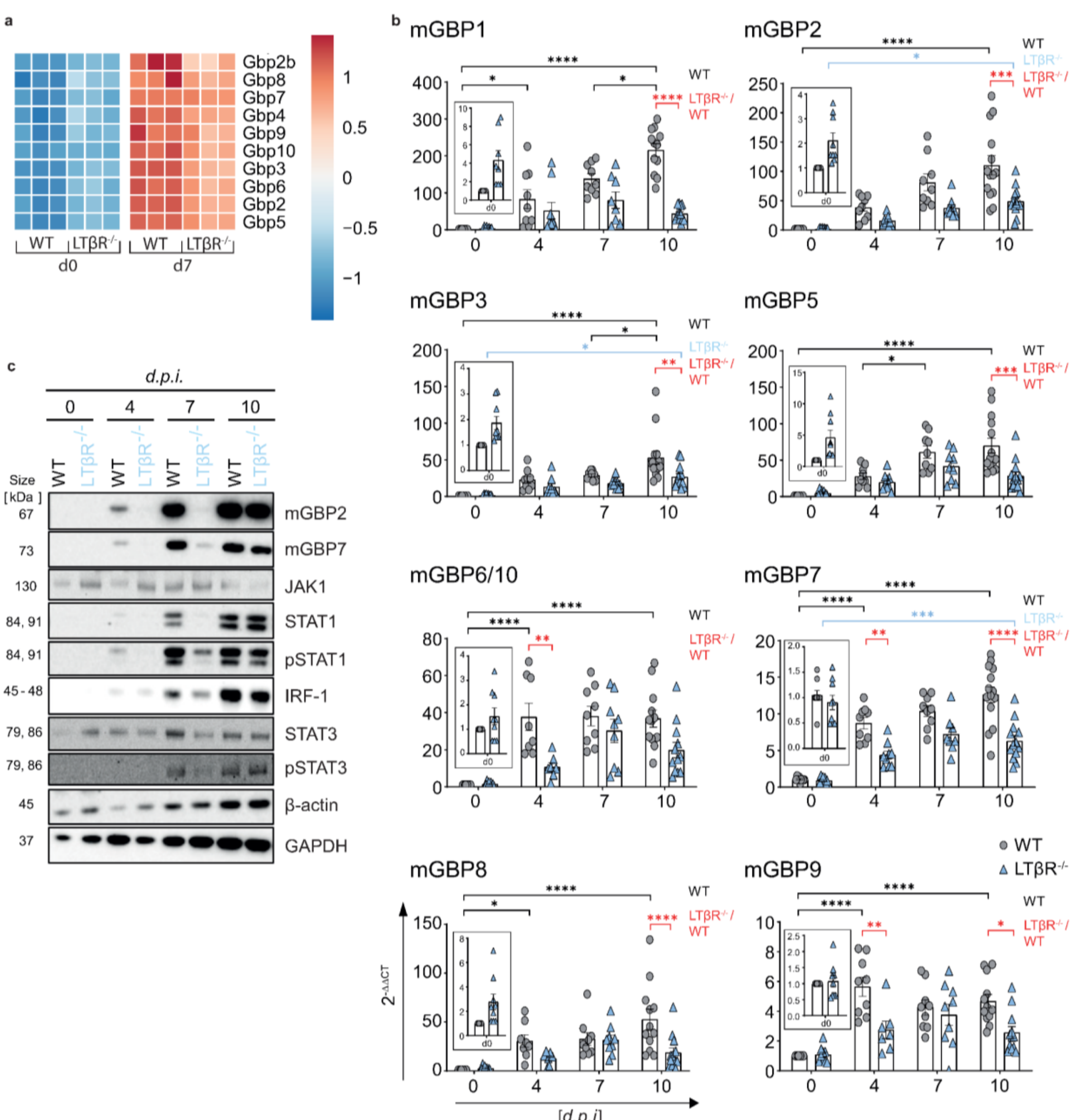
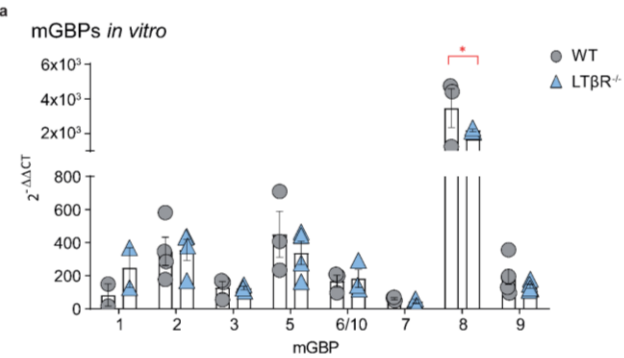


FIG 3 LTβR deficiency dysregulates IFN-γ signaling in the lung. (a) Heat map of differentially expressed murine guanylate-binding proteins (mGBPs) based on RNA-seq analysis (Wald test and adjusted *P* value of 0.1) of lung tissue from uninfected (d0) and *T. gondii*-infected (ME49, 40 cysts i.p., d7 p.i.) WT and LTβR$^{-/-}$ mice ($n = 3$). (b) qRT-PCR of mGBPs in lung tissue from uninfected and *T. gondii*-infected WT and LTβR$^{-/-}$ mice (d0 to 7, $n \geq 12$; d10, $n \geq 14$). The data shown represent four independent experiments; symbols represent individual animals, columns represent mean values, and error bars represent the ± SEM. (c) Immunoblot analysis of proteins involved in or induced via the IFN-γ signaling pathway in lung tissue from uninfected and *T. gondii*-infected WT and LTβR$^{-/-}$ mice. Two-way ANOVA corrected for multiple comparison using Tukey's *post hoc* test was used for the statistical analysis represented in panel b. *, $P < 0.0332$; **, $P < 0.0021$; ***, $P < 0.0002$; ****, $P < 0.0001$. The data shown in panel c are representative of three independent experiments.

To summarize, mRNA and protein expression data from the lungs indicate that uninfected LTβR$^{-/-}$ animals show an activated immune status compared to WT animals but fail to adequately upregulate IFN-γ-dependent immune effector responses after *T. gondii* infection, possibly explaining the increased parasite burden and the subsequently increased infection susceptibility of LTβR$^{-/-}$ mice.



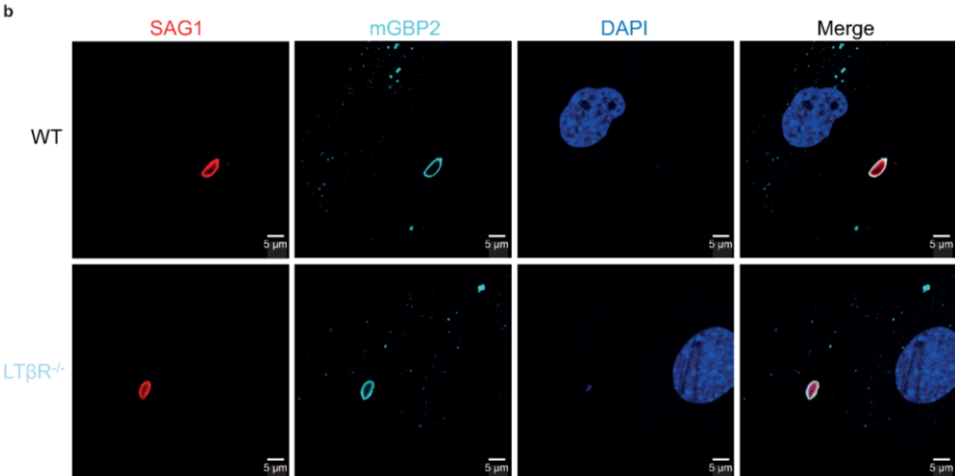


FIG 4 mGBP upregulation and recruitment. (a) qRT-PCR analysis of mGBP mRNA expression of uninfected WT and LTβR$^{-/-}$ MEFs stimulated with IFN-γ (7.5 ng/ml) for 8 h (all $n = 3$, except for mGBP1, where $n = 2$). Each symbol represents an individual technical replicate; columns represent mean values, and error bars represent the ± SEM. two-way ANOVA corrected for multiple comparisons by the Sidak *post hoc* test was used for statistical analysis. *, $P < 0.00332$. (b) Representative immunofluorescence analysis of *T. gondii* tachyzoite-infected (multiplicity of infection [MOI], 1:40) WT and LTβR$^{-/-}$ MEFs. Cells were prestimulated with IFN-γ (7.5 ng/ml) for 16 h before being infected with *T. gondii* tachyzoites for 2 h. *T. gondii* surface antigen SAG1 was visualized using a Cy3-conjugated secondary antibody, and mGBP2 was visualized using an mGBP2 antiserum (30) followed by an Alexa Fluor 633-conjugated secondary antibody for detection of mGBP2 recruitment toward the *T. gondii* PV. Cell nuclei were stained using DAPI (4',6-diamidino-2-phenylindole). The data shown in panels a and b represent at least two independent experiments.

mGBP upregulation and recruitment to the PV after IFN-γ stimulation *in vitro*. Since upregulation of mGBP expression was impaired in LTβR$^{-/-}$ mice after *T. gondii* infection (Fig. 3), we asked whether IFN-γ-dependent upregulation of mGBP expression was directly dependent on LTβR signaling (Fig. 4). We therefore analyzed whether LTβR$^{-/-}$ mouse embryonic fibroblasts (MEFs) were able to upregulate mGBPs after IFN-γ stimulation and whether mGBPs could recruit to the PV in infected, IFN-γ-pre-treated LTβR$^{-/-}$ MEFs. After preincubation with IFN-γ *in vitro*, *T. gondii*-infected LTβR$^{-/-}$ and WT MEFs showed comparable upregulation of all tested mGBPs (mGBP1, 2, 3, 5, 6/10, 7, and 9), with the exception of mGBP8, where WT mice showed increased mRNA expression (Fig. 4a). Also, after preincubation with IFN-γ, mGBP2 was recruited to the PV of *T. gondii* in LTβR$^{-/-}$ MEFs (Fig. 4b). These results demonstrate that expression of mGBPs can be successfully induced in LTβR$^{-/-}$ MEFs in the presence of exogenous IFN-γ and that the lack of LTβR signaling appears not to interfere with the ability of mGBP2 to recruit to the PV in LTβR$^{-/-}$ MEFs. This suggests that the absence of LTβR signals do not impact IFN-γR signaling required for mGBP function.

Differences in spleen size and weight in LTβR$^{-/-}$ mice. Since LTβR$^{-/-}$ mice lack lymph nodes (7), the spleen is the primary organ where the immune response against *T. gondii* is primed. It has been described that during the acute phase of *T. gondii* infection, the splenic architecture is disrupted transiently (39). When we compared spleens of WT versus LTβR$^{-/-}$ mice, spleens of the latter were markedly larger (Fig. S5a) in uninfected (day 0) healthy animals. While spleens of both genotypes significantly increased in weight during the course of *T. gondii* infection, spleen weights of WT mice were significantly higher than those of LTβR$^{-/-}$ mice on day 10 p.i. (Fig. S5b). This increase of spleen weight in WT mice was not due to increased cellularity, as splenocyte counts were consistently higher in LTβR$^{-/-}$ spleens before as well as on days 4 and 7 p.i. (Fig. S5c). By day 10 p.i., cell numbers in the spleens of both genotypes were comparable, mostly due to a significant drop of splenocyte numbers in LTβR$^{-/-}$ mice. This also indicates that the initial immune response in spleens of LTβR$^{-/-}$ mice is disturbed.

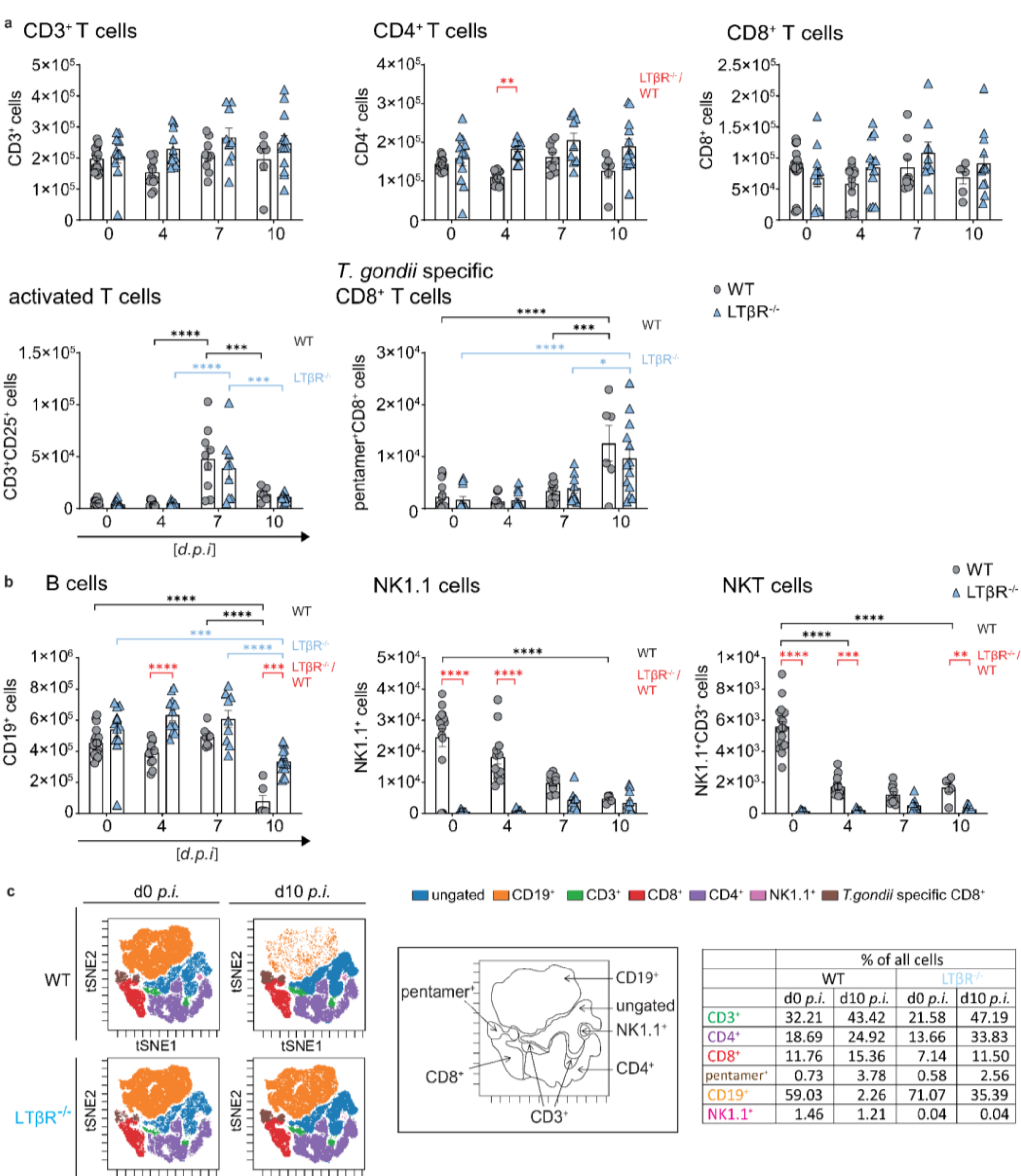
No apparent difference in T cell subpopulations in spleens of LTβR$^{-/-}$ mice. Accordingly, we analyzed the composition of the splenocytes using flow cytometry (Fig. 5). Since T cells are essential to control *T. gondii* infection (49, 50), we analyzed T cell subpopulations in LTβR$^{-/-}$ spleens (Fig. 5a). Analysis of absolute numbers of CD3$^+$, CD4$^+$, CD8$^+$, activated (CD3$^+$CD25$^+$) T cells, and *T. gondii*-specific (pentamer$^+$) CD8$^+$ T cells (Fig. 5a) revealed almost no significant differences between WT and LTβR$^{-/-}$ mice either before or during infection. The only exception was CD4$^+$ T cells on day 4 p.i., where WT and LTβR$^{-/-}$ mice showed a moderate decrease and increase, respectively. In both genotypes, the numbers of activated CD3$^+$CD25$^+$ T cells were significantly increased on day 7 p.i., but LTβR$^{-/-}$ mice showed similar numbers of total T cells. In addition, LTβR$^{-/-}$ mice showed a comparable rise of activated CD3$^+$CD25$^+$ T cells on day 7 p.i. and a comparable expansion of *T. gondii*-specific (pentamer$^+$) CD8$^+$ T cells on day 10 p.i. (Fig. 5a).

Baseline numbers of CD19$^+$ B cells were somewhat higher in LTβR$^{-/-}$ mice and significantly increased on day 4 p.i., but while numbers of CD19$^+$ B cells dropped significantly in both genotypes on day 10 p.i., they were still significantly higher in LTβR$^{-/-}$ mice (Fig. 5b).

Since LTβR$^{-/-}$ mice are known to have fewer NK cells and NKT cells (8, 51, 52), it was not surprising to observe that absolute NK1.1$^+$ cells were significantly higher in WT than in LTβR$^{-/-}$ mice before infection and on days 4 and 7 p.i. (Fig. 5b). On day 10 p.i., NK1.1$^+$ cell numbers of both genotypes were similar, due to the drop of NK1.1$^+$ cells in spleens of WT mice during the course of infection. Similarly, the absolute numbers of NK1.1$^+$CD3$^+$ NKT cells in WT mice declined during the course of infection but were higher than those of LTβR$^{-/-}$ mice before infection and on days 4 and 7 p.i., which is in accordance with published data (51). Unbiased analysis of the cytometry data set using t-distributed stochastic neighbor embedding (tSNE) (Fig. 5c) confirmed these data, notably the absence of NK1.1$^+$ cells in uninfected LTβR$^{-/-}$ mice (1.46% and 0.04%, respectively) and the marked drop in absolute CD19$^+$ B cell numbers in WT mice by day 10 p.i. which was absent in LTβR$^{-/-}$ animals (59.03% to 2,26% versus 71.07% to 35.39%, respectively; Fig. 5c). This demonstrates that the deficiency of the LTβR does not impact T cell numbers after *T. gondii* infection, especially the expansion of parasite-specific T cells, while it does seem to influence B cell numbers during the acute phase of *T. gondii* infection.

In conclusion, LTβR$^{-/-}$ compared to WT mice do not show a significant difference in overall and antigen-specific T cell numbers either before or after *T. gondii* infection, but B cell, NK1.1$^+$, and NKT cell numbers appear to be significantly affected by the absence of LTβR before and during infection.

Impaired T cell effector function in the spleen in the absence of the LTβR. Even though LTβR$^{-/-}$ mice are highly susceptible to *T. gondii* infection, we detected comparable CD8$^+$ and *T. gondii*-specific CD8$^+$ T cell numbers in the spleen (Fig. 5a). We therefore decided to determine whether these T cells were fully differentiated and functional with regard to their ability to produce IFN-γ, contained cytotoxic granules (GzmB$^+$ and perforin$^+$),



	% of all cells			
	WT		LTβR-/-	
	d0 *p.i.*	d10 *p.i.*	d0 *p.i.*	d10 *p.i.*
CD3⁺	32.21	43.42	21.58	47.19
CD4⁺	18.69	24.92	13.66	33.83
CD8⁺	11.76	15.36	7.14	11.50
pentamer⁺	0.73	3.78	0.58	2.56
CD19⁺	59.03	2.26	71.07	35.39
NK1.1⁺	1.46	1.21	0.04	0.04

FIG 5 Dysregulated immune cell numbers in LTβR$^{-/-}$ mice. (a) Absolute cell numbers of CD3$^+$, CD4$^+$, CD8$^+$, CD25$^+$CD3$^+$, and pentamer$^+$CD8$^+$ T cells. (b) CD19$^+$, NK1.1$^+$, and NK1.1$^+$CD3$^+$ cells in spleens of uninfected (d0) and *T. gondii*-infected (ME49, 40 cysts, i.p.) WT and LTβR$^{-/-}$ mice (d0 to d7 p.i., $n = 12$; d10 p.i., $n \geq 6$) determined via flow cytometry. (c) Representative tSNE plots from splenocytes of uninfected and *T. gondii*-infected (d10 p.i.) WT and LTβR$^{-/-}$ mice. Clustered populations were identified using the indicated markers. The data shown represent at least three independent experiments; symbols represent individual animals, columns represent mean values, and error bars represent the ± SEM. Two-way ANOVA corrected for multiple comparison using Tukey's *post hoc* test was used for the statistical analysis represented in panels a and b. *, $P < 0.0332$; **, $P < 0.0021$; ***, $P < 0.0002$; ****, $P < 0.0001$.

and were able to degranulate (CD107a$^+$ cells) upon stimulation. In order to address this question, splenocytes of infected WT and LTβR$^{-/-}$ mice (day 7 and 10 p.i.) were prepared and were restimulated *ex vivo* with toxoplasma lysate antigen (TLA) before flow cytometry analysis (Fig. 6).

After *ex vivo* TLA restimulation, LTβR$^{-/-}$ T cells compared to WT T cells showed a significantly reduced frequency of CD4$^+$ IFN-γ-producing T cells in splenocytes from

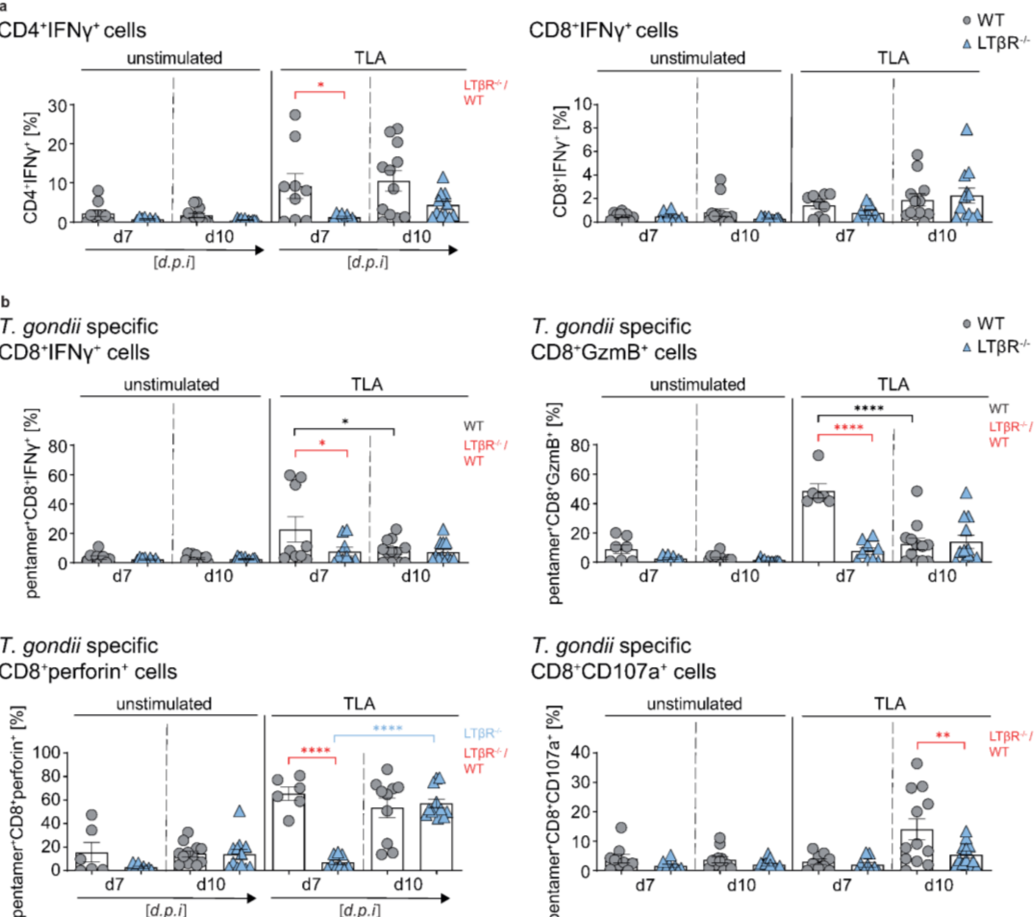


FIG 6 LTβR deficiency impairs T cell effector function in the spleen. (a and b) Intracellular staining of (a) $CD4^+$ IFN-γ^+ and $CD8^+$ IFN-γ^+ T cells (%) and (b) cytotoxic granule ($GzmB^+$ or $perforin^+$) containing and degranulating ($CD107a^+$) $pentamer^+CD8^+$ T cells of unstimulated and toxoplasma lysate antigen (TLA) *ex vivo* restimulated splenocytes from *T. gondii*-infected (d7 and 10 p.i.) WT and $LT\beta R^{-/-}$ mice (d7, $n \geq 6$; d10, $n \geq 10$). Representative data of at least two independent experiments; symbols represent individual animals, columns represent mean values, and error bars represent the ± SEM. Two-way ANOVA corrected for multiple comparison using Tukey's *post hoc* test was used for statistical analysis. *, $P < 0.0332$; **, $P < 0.0021$; ****, $P < 0.0001$.

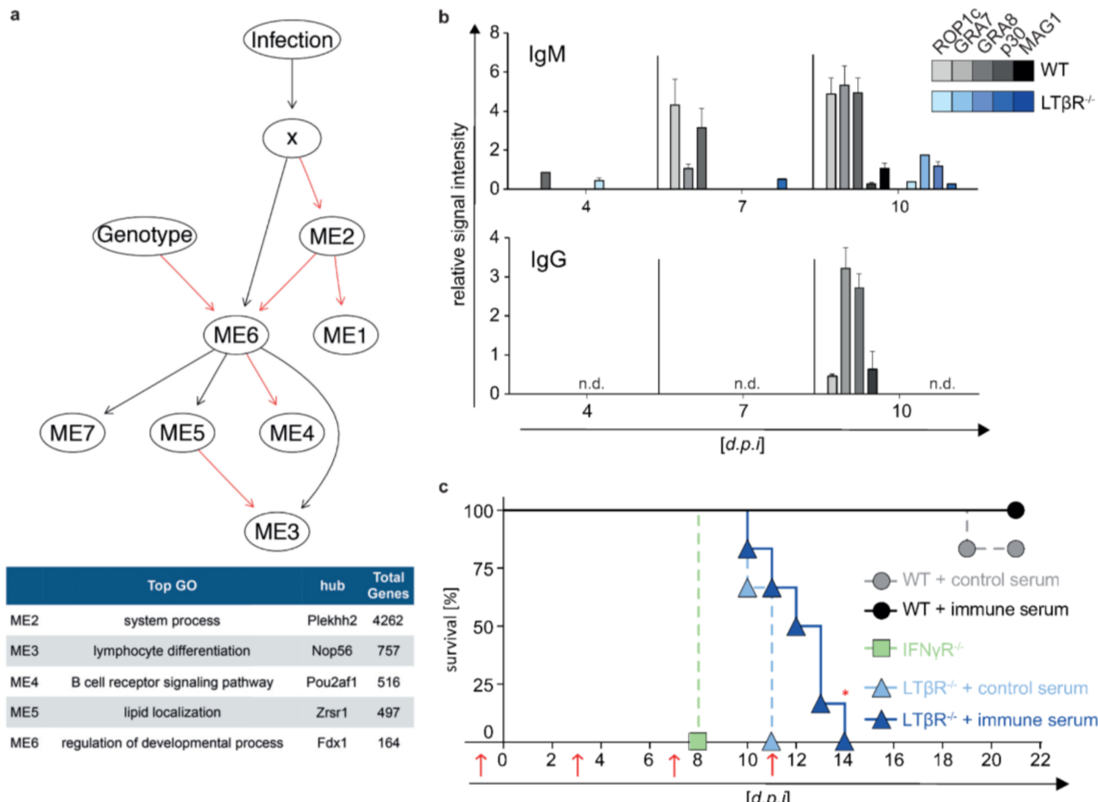
day 7 p.i. and a reduced percentage in splenocytes at day 10 p.i. Similar frequencies for $CD8^+$ IFN-γ-producing T cells could be detected in restimulated splenocytes for both genotypes on both days (Fig. 6a). There were no significant differences between the two genotypes for granzyme B-containing $CD8^+$ cells in restimulated cells from either day 7 or day 10 p.i. (Fig. S6a). For $CD8^+perforin^+$ cells, WT mice showed higher frequencies in day 10 restimulated cells, but $LT\beta R^{-/-}$ mice showed a delayed but significant increase from day 7 to day 10, resulting in frequencies similar to those of WT mice for day 10 p.i. (Fig. S6a). In contrast, $CD8^+107a^+$ T cell frequencies in WT spleens increased significantly in restimulated cells from day 10 p.i. and were significantly higher than those of $LT\beta R^{-/-}$ spleens (Fig. S6a). When we directly analyzed IFN-γ^+GzmB^+ and IFN-$\gamma^+perforin^+$ cells, we found a significantly higher frequency in restimulated splenocytes of WT mice at day 7 p.i. than those of $LT\beta R^{-/-}$ mice (Fig. S6b).

For *T. gondii*-specific ($pentamer^+$) $CD8^+$ T cells (Fig. 6b), we found a significantly higher frequency of $pentamer^+CD8^+$ IFN-γ-producing T cells in restimulated splenocytes from WT mice on day 7 p.i. compared to $LT\beta R^{-/-}$ mice but not in restimulated splenocytes from day 10 p.i. Interestingly, *T. gondii*-specific $CD8^+GzmB^+$ T cells showed

a similar picture—a significantly increased frequency in restimulated splenocytes from WT mice on day 7 p.i. compared to LTβR$^{-/-}$ mice and no difference of these cells in splenocytes from day 10 p.i. In WT compared to LTβR$^{-/-}$ spleens, *T. gondii*-specific CD8$^+$perforin$^+$ T cells were also significantly higher in restimulated WT splenocytes at day 7 p.i. However, here, LTβR mice showed a significantly increased frequency of CD8$^+$perforin$^+$ T cells in restimulated splenocytes from day 10 compared to day 7 p.i., resulting in similar frequencies for WT and LTβR$^{-/-}$ CD8$^+$perforin$^+$ T cells at day 10 p.i. Finally, the percentage of *T. gondii*-specific CD8$^+$CD107a$^+$ T cells was similar for both genotypes in restimulated splenocytes at day 7 p.i. but significantly increased for WT mice in restimulated splenocytes from day 10 p.i., whereas only few CD8$^+$ LTβR$^{-/-}$ T cells degranulated. To summarize, importantly, parasite-specific granzyme B granule containing (pentamer$^+$CD8$^+$GzmB$^+$) as well as degranulating (pentamer$^+$CD8$^+$CD107a$^+$) T cells do not appear to be detectable in LTβR$^{-/-}$ mice after *T. gondii* infection, whereas the increase of parasite-specific perforin granule-containing (pentamer$^+$CD8$^+$perforin$^+$) T cells seems to be delayed in LTβR$^{-/-}$ compared to WT mice. These results demonstrate that while the T cell compartment does not seem to be affected in regard to cell numbers, LTβR$^{-/-}$ mice show a clear functional defect in the parasite-specific CD8$^+$ T cell compartment as well as clearly decreased IFN-γ-producing CD4$^+$ T cells after infection.

LTβR deficiency abrogates *T. gondii*-specific isotype class switching. RNA-seq data of lung tissue from uninfected (day 0) and *T. gondii*-infected (day 7 p.i.) WT and LTβR$^{-/-}$ animals were further analyzed to elucidate the interaction between *T. gondii* and host immune responses. The data were filtered for differentially expressed genes, and hierarchical clustering was performed and illustrated as a sample dendrogram with a trait heat map (Fig. S7a) for identification of possible outliers. All tested samples showed adequate clustering and could, accordingly, be grouped into uninfected and infected WT and LTβR$^{-/-}$ mice. Next, gene expression data were condensed into 10 module eigengenes (ME0 to ME9; Fig. S7b) and used to generate a host-pathogen network prediction model (Fig. 7a) displaying the relationship between modules (ME) and experimental conditions. This model captures the influence of *T. gondii* infection ("Infection" in the figure), the LTβR$^{-/-}$ genotype ("Genotype"), and total *T. gondii* genes ("X") on host gene modules (ME0 to ME9) detected in each sample. Upon closer inspection, this model shows that LTβR expression (contained in ME6) is suppressed by the LTβR$^{-/-}$ genotype, which fits our experimental conditions. This model predicts that in WT mice, high expression of genes contained in ME6 suppresses genes contained in ME4 (top GO term "B cell receptor signaling pathway") while enhancing gene expression in ME3 (top GO term "lymphocyte differentiation"). This implies that the loss of the LTβR slightly increases ME4 levels (Fig. S7c; top GO term "B cell receptor signaling pathway"; Fig. 7a) containing genes for "immunoglobulin production" and "humoral immune response mediated by circulating immunoglobulin" during *T. gondii* infection. Furthermore, the network predicts that in LTβR$^{-/-}$ mice, *T. gondii* infection reduces ME3 levels (Fig. S7d; top GO term "lymphocyte differentiation"; Fig. 7a) containing genes for "B cell activation" and "isotype switching." In addition, GSEA generated from RNA-seq data also showed significant upregulation of these pathways, indicating a disturbed B cell response (Fig. S2).

Due to this highly surprising prediction, as well as the different B cell numbers of WT and LTβR$^{-/-}$ mice in the spleen on day 10 p.i. (Fig. 5a and c), we then asked whether an altered B cell-mediated humoral immune response could be directly involved in the high mortality of LTβR$^{-/-}$ mice after *T. gondii* infection. The presence of immunoglobulin M (IgM) and IgG antibodies specific for *T. gondii* antigens was determined during the acute phase of infection (days 4, 7, and 10 p.i.) using line blots coated with specific recombinant *T. gondii* tachyzoite and bradyzoite antigens (ROP1c, GRA7, GRA8, p30, and MAG1). LTβR$^{-/-}$ mice compared to WT mice showed a delayed and reduced *T. gondii*-specific IgM and, surprisingly, an abrogated *T. gondii*-specific IgG antibody response in the serum during infection (days 4, 7, and 10 p.i.; Fig. 7b),



	Top GO	hub	Total Genes
ME2	system process	Plekhh2	4262
ME3	lymphocyte differentiation	Nop56	757
ME4	B cell receptor signaling pathway	Pou2af1	516
ME5	lipid localization	Zrsr1	497
ME6	regulation of developmental process	Fdx1	164

FIG 7 Abrogated parasite-specific isotype class switching and reconstitution of mice with *T. gondii*-specific immune serum. (a) Host-pathogen network prediction model generated based on RNA-seq data of lung tissue of uninfected (d0) and *T. gondii*-infected (ME49, 40 cysts; d7 p.i.) WT and LTβR$^{-/-}$ mice ($n=3$/group). GmicR was used to detect relationships between module eigengenes (ME) and experimental conditions. X represents the total *T. gondii* gene expression data for each sample; infection and genotype were included as variables. Red lines indicate inverse and black lines positive relationships. Representative gene ontologies and hub genes reported by GmicR for each module are shown in the summary table. (b) *T. gondii*-specific IgM and IgG antibody response in serum of uninfected (d0) and *T. gondii*-infected (ME49, 40 cysts, i.p.) WT and LTβR$^{-/-}$ mice (d4 and d7 p.i., $n=15$; d10 p.i., $n \geq 20$). Shown is a representative result of four independent experiments; bars represent mean values $\pm$ SEM. (c) Transfer of serum (red arrows; d1, d3, d7, and d11 p.i.) from uninfected donor WT mice (control serum) or from *T. gondii*-infected (ME49, 20 cysts, i.p.) donor WT mice (immune serum) into WT and LTβR$^{-/-}$ acceptor mice. On day 0, acceptor mice ($n=6$/group) were infected with *T. gondii* (ME49, 10 cysts, i.p.), and survival was evaluated. IFN-γR$^{-/-}$ mice ($n=3$) served as infection controls. The data shown in panel c represent one experiment. A log rank (Mantel Cox) test was used for the statistical analysis represented in panel c. *, $P < 0.0332$; n.d., not detected.

demonstrating a lack of functional isotype switching that is in line with the bioinformatic host-pathogen prediction network.

LTβR deficiency can be partially compensated for by transfer of *T. gondii* immune serum. Since it has been described that a *T. gondii*-specific IgG response is required for a reduction of the parasite burden (25, 51), we treated LTβR$^{-/-}$ mice with serum from *T. gondii*-infected WT animals (immune serum) and uninfected mice (control serum) and monitored survival after *T. gondii* infection (Fig. 7c). Serum transfer experiments showed that LTβR$^{-/-}$ mice treated with immune serum exhibit significantly prolonged survival (up to day 14 p.i.) compared to littermates that received control serum, which died by day 11 p.i. IFN-γR$^{-/-}$ mice served as infection controls and succumbed as reported around day 8 p.i. (53). These data demonstrate that LTβR-mediated signaling is essential for the development of an efficient humoral immune response to *T. gondii* infection.

DISCUSSION

The results obtained in this study corroborate a profoundly deficient immune response of LTβR$^{-/-}$ mice to *T. gondii* infection and reveal an impaired IFN response, a

severe functional T cell defect, as well as a humoral immune deficiency in the absence of LTβR.

One reason for the significantly increased parasite burden and significantly reduced survival rates of LTβR$^{-/-}$ mice is the inadequate cytokine, especially the IFN-γ, response. The elevated levels of LTα and significantly increased levels of LTβ in the lung of LTβR$^{-/-}$ mice could be caused by compensatory mechanisms and/or lack of negative feedback mechanisms due to the absence of the LTβR. Since we also found elevated levels for IFN-γ, IL-6, IFN-β, IL-1α, and IL-17A and significantly elevated expression levels for IL-1β in the serum and for IL-4 in the lung, we suggest that, overall, uninfected LTβR$^{-/-}$ mice show a dysregulated, more activated, albeit stable immune homeostasis. This is in accordance with the finding that LTβR$^{-/-}$ animals present with splenomegaly, probably due to microbiota-mediated inflammation (54). When *T. gondii* infection disrupts this precarious balance in LTβR$^{-/-}$ mice, the dysregulation becomes more pronounced; on the one hand, LTβR$^{-/-}$ mice have lower levels of IFN-γ in the serum early during infection, but on day 10 p.i., when WT mice already show decreased IFN-γ levels, they remain high in LTβR$^{-/-}$ mice, not only in serum but also in the lungs. Conversely, IL-6 expression in the serum is markedly increased in LTβR$^{-/-}$ mice compared to WT mice throughout the infection. This suggests that by day 10 p.i., parasite expansion is being controlled in WT but not LTβR$^{-/-}$ mice. The significantly increased levels of IL-10 in LTβR mice on day 10 *p.i.* could be a protective/counteractive mechanism to prevent extensive immunopathology (55). Interestingly, several cytokines in LTβR$^{-/-}$ mice are transiently but significantly upregulated on day 4 p.i. This also suggests a disruption of the precarious immune homeostasis in LTβR mice. In contrast to the activated immune homeostasis in LTβR$^{-/-}$ mice, they show decreased expression levels for chemokines/chemokine receptors, genes involved in IFN-γ signaling, and IFN-γ-induced genes in the lung on day 7 p.i. This points toward an inability of LTβR$^{-/-}$ mice to mount an efficient immune response to *T. gondii* infection and is supported by the finding that upregulation of IFN-γ-regulated effector molecules known to be important for *T. gondii* containment, such as iNOS, IDO1, NOX2-gp91phox, and mGBPs, is deficient in LTβR animals. In the lung, in the case of NOX2-gp91phox, this could be due to the lack of TNF-α expression, as it has been shown in *ex vivo* experiments of bronchoalveolar fluid cells and human pulmonary artery endothelial cells that TNF-α upregulates NOX2-g91phox (56, 57). The mRNA expression profile of mGBPs and the protein expression of mGBPs 2 and 7 also fit into this pattern; mGBPs are essential for efficient control of *T. gondii* expansion (31, 33, 58), and RNA-seq analysis shows that uninfected LTβR mice have overall increased expression of, while infected animals show overall less upregulation. And while LTβR$^{-/-}$ animals do upregulate mGBP expression during the course of infection, they show significantly lower expression on day 10 p.i. than WT mice in all cases except for mGBP6/10.

LTβR$^{-/-}$ animals also show increased baseline expression of IFN-γ mRNA in the lung, which would explain the elevated baseline JAK1 protein expression. Increased JAK expression should lead to increased JAK phosphorylation and, consequently, increased STAT1 recruitment and STAT1 phosphorylation (59, 60). However, we observed delayed upregulation of STAT1 and less pSTAT1 protein in infected LTβR$^{-/-}$ animals and therefore hypothesize that the lack of LTβR signaling somehow affects STAT1 expression or recruitment via a so far unknown mechanism. Notably, Kutsch et al. also showed reduced STAT1 expression in LTβR$^{-/-}$ mice (61).

We conclude that, due to the underlying dysregulation of the immune homeostasis, LTβR$^{-/-}$ mice are unable to initiate a coordinated immune response, leading to either delayed upregulation of essential cytokines (e.g., IFN-γ) or overexpression of others (e.g., IL-6, TNF). This is also supported by our findings that LTβR$^{-/-}$ mice do not show the typical splenomegaly associated with (*T. gondii*) infection (39).

In line with published data, we found a virtual absence of NK1.1^{+} cells in LTβR$^{-/-}$ mice, and NK1.1^{+} cell numbers dropped in WT mice after infection, probably due to conversion into ILCs (51). Also, a lack of NKT cells has been shown for LTβR$^{-/-}$ mice

(62). Interestingly, a dual role for NKT cells in *T. gondii* infection has been described; on the one hand, they release large amounts of IL-4 and IFN-γ upon activation to shift the T cell response toward a Th1 pattern, and on the other hand, the uncontrolled Th1 response can lead to severe immunopathology (63). Since they have also been indicated in the suppression of a protective immunity against *T. gondii* infection (64), it is maybe not surprising that their numbers are downregulated after infection in WT animals.

Overall, we did not find T cell numbers to be significantly different in either uninfected or *T. gondii*-infected LTβR$^{-/-}$ mice compared to WT mice. However, we found profound defects in T cell effector functions; the reduced number of IFN-γ-producing CD4^{+} T cells and functional *T. gondii*-specific CD8^{+} cytotoxic lymphocytes (GzmB^{+}, perforin+, CD107a^{+}) strongly implies that cytotoxic T cell-mediated killing is severely impaired in LTβR$^{-/-}$ animals. Since these responses are known to be essential for efficient *T. gondii* containment, this marked functional deficiency is probably one reason for the susceptibility of LTβR$^{-/-}$ mice to the parasite.

In contrast to T cell numbers, B cell numbers differed significantly in LTβR$^{-/-}$ mice compared to WT mice. On day 10 postinfection, the numbers of CD19^{+} B cells in WT spleens were significantly lower than those of LTβR$^{-/-}$ animals. This is probably due to maturation of B cells to IgG-producing plasma cells in WT mice, which emigrate to the bone marrow and lose surface CD19 in the process. In LTβR$^{-/-}$ mice, the lack of class switching would inhibit maturation and migration of B cells to the bone marrow.

Since the host-pathogen network prediction model we generated from *T. gondii*-infected mice indicated that the loss of the LTβR inhibits B cell responses, including isotype switching in *T. gondii* infection, we further analyzed the humoral immune response. We demonstrated that *T. gondii*-infected LTβR$^{-/-}$ mice produced less *T. gondii*-specific IgM than WT mice, and no detectable *T. gondii*-specific IgG. Whether this failure is due to impaired IFN-γ production which is an important cytokine for isotype class switching (65) will be determined in the future.

While Glatman Zaretsky et al. (39) argue that the disrupted lymphoid structure, which includes the lack of defined germinal centers in LTβR$^{-/-}$ mice, is the main cause of the reduced antibody response, Ehlers et al. (5) show via bone marrow (BM) chimeras that the effects of LTβR deficiency in *M. tuberculosis* infection cannot be attributed solely to the architectural differences but are also directly caused by the lack of LTβR-mediated signaling. LTα, another member of the TNF/TNFR superfamily, has similar but not identical functions to LTβ in the development of secondary lymphoid organs and immune modulation (2). LT$\alpha^{-/-}$ animals also present with a disturbed architecture of the lymphoid system (no LNs, no PPs, no germinal centers, and a disorganized white pulp) (2, 66). *T. gondii*-infected LT$\alpha^{-/-}$ mice are shown to have reduced numbers of *T. gondii*-specific IFN-γ-producing T cells and lower *T. gondii*-specific antibody titers, but BM chimera experiments demonstrated that an intact secondary lymphoid system is not sufficient to generate an effective immune response (25). Interestingly, the study of Glatman Zaretsky et al. (39), which uses the *T. gondii* Prugniaud strain, also a type II strain, found parasite-specific IgM in serum as early as day 7 postinfection in WT animals, which is in line with our observations in this study. This indicates a comparable timeline in the induction of immune responses and antiparasitic effector mechanisms in WT animals to *T. gondii* in the two type II strains. We therefore feel confident that our results are representative of the infections with a type II strain, which are the prevalent strains in human toxoplasmosis in North America and Europe (67–70).

Although protective B cell responses have been described to play a more significant role in chronic rather than acute *T. gondii* infection in some *T. gondii* infection models (49–51, 71), our data indicate that a robust humoral immune response is dependent on LTβR signaling and is a prerequisite for survival during acute *T. gondii* infection. This conclusion is validated by our data showing that the survival of LTβR$^{-/-}$ animals can be significantly prolonged by transfer of immune serum containing *T. gondii*-specific antibodies.

Finally, the host-pathogen prediction network generated in this study indicates that *T. gondii* infection suppresses B cell responses in WT animals. This could point toward an unknown *T. gondii* strategy to evade the host immune system. Early *T. gondii*-mediated suppression of B cell responses could support dissemination and cyst formation in the brain, facilitating the establishment of chronic infection (25, 72). Since *T. gondii* is known to have developed different mechanisms to evade host immune responses (73), it is worth exploring this approach in the future.

We demonstrate that the loss of LTβR signaling results in a combined and profoundly depressed IFN-γ response, impaired T cell functionality, and the failure to induce parasite-specific IgG antibodies, leading to an increase in parasite burden and fatal outcome of *T. gondii* infection. Therefore, for the first time, we suggest an LTβR-mediated modulation of the IFN-γ signaling pathway *in vivo*. Further understanding of this complex interplay between LTβR and IFN-γ signaling pathways will provide new insights into the pathogenesis of *T. gondii* and may provide novel therapeutic strategies.

MATERIALS AND METHODS

Mice. LTβR $^{/}$ mice were previously described (7) and are back-crossed for at least 10 generations onto a C57BL/6N background. Wild-type (WT) littermates were used as controls. Mice were kept under specific-pathogen-free (SPF) conditions in the animal facility at the Heinrich Heine University Düsseldorf and were 8 to 16 weeks old for experiments. Cysts of the ME49 strain (substrain 2017) of *T. gondii* were collected from the brain tissue of chronically infected CD1 mice. All animal experiments were conducted in strict accordance with the German Animal Welfare Act. The protocols were approved by the local authorities (Permit no. 84-02.04.2013.A495, 81-02.04.2018.A406, and 81-02.05.40.18.082). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

***Toxoplasma gondii* infection experiments.** Mice were intraperitoneally (i.p.) infected with 40 cysts (ME49 strain) and weighed and scored daily for the duration of the experiments. Mice were euthanized on days 4, 7, and 10 postinfection (dpi); uninfected mice (d0) served as controls. After euthanasia (100 mg/kg ketamine, 10 mg/kg xylazine; Vétoquinol GmbH), blood was taken from the vena cava inferior, and spleen, lung, and muscle tissue was harvested for analysis.

Detection of parasite load. Total DNA was isolated from tissues using a DNA isolation kit (Genekam) according to the manufacturer's protocol. qRT-PCR was performed on a Bio-Rad CFX-96 Touch real-time detection system. To determine parasite load, PCR of DNA isolated from defined numbers (101 to 105) of *in vitro*-cultured ME49 tachyzoites (using distilled water [dH_2O] as a negative control) was performed to generate a standard curve; TgB1 primers and probe (Metabion) were used to amplify a defined sequence of the 35-fold repetitive B1 gene from *T. gondii* and are listed in Table S1. This *T. gondii* standard curve was used to determine B1 amplification for calculation of parasite load.

Cytokine measurement. Cytokines CCL2, IFN-γ, IFN-β, IL-1α, IL-1β, IL-6, IL-10, IL12p70, IL-17A, IL-23, IL-27, and TNF-α were measured using the LEGENDplex mouse inflammation panel (BioLegend) according to the manufacturer's protocol. Samples were measured using a BD FACSCanto II instrument.

Real-time qRT-PCR. Total RNA was isolated from tissues using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was reverse-transcribed using SuperScript III reverse transcriptase (200 U/μl; Invitrogen). qRT-PCR was performed on the Bio-Rad CFX-96 Touch real-time detection system. The primer sequences and corresponding probes (Metabion, Roche, and TIB Molbiol) are listed in Table S1. Results are expressed relative to expression in untreated WT mice normalized to β-actin ($2^{-\Delta\Delta CT}$).

RNA-seq analysis. Lung tissue of uninfected (d0) and *T. gondii*-infected (ME49 strain, 40 cysts, i.p.) WT and LTβR$^{-/-}$ mice was obtained, and RNA sequencing was performed on a HiSeq 3000 device. Mouse and *T. gondii* transcripts were quantified from FASTQ files using Salmon with default settings and GC bias compensation. For transcriptome models, *Mus musculus* GRCm38 cDNA (ensembl.org, release-97) and *Tgondii*ME49 annotated transcripts (toxodb.org, ToxoDB-45) were used. Mouse transcripts from pseudogenes or with retained introns were excluded prior to conversion to gene counts using the DESeq2 package. Non-protein-encoding *T. gondii* transcripts were excluded prior to conversion to gene counts. DEseq2 was used to test for genotype-specific responsiveness to infection with the following model: ~genotype · infection. To calculate WT-specific responsiveness, we used the following model: ~genotype + genotype: infection. For significance, the Wald test with an adjusted *P* value of 0.1 was used.

Host-pathogen network generation. Previously developed analytic tools for 'omics data sets were used to generate the host-pathogen network as described (74). Prior to network generation, the variance stabilizing transformation (VST)-normalized data were filtered for genes that showed significant differential expression for at least one contrast. This produced an expression matrix for 10,748 genes. The GmicR package was then used for module detection, using a minimum module size of 30, mergeCutHeight of 0.3, and Rsquared cut of 0.80. To detect relationships between modules and infection, VST-normalized data *T. gondii* expression levels for each sample were aggregated by sum, and then these numeric data were merged to module eigengenes using the Data_Prep function of GmicR (Fig. S6). Genotype and infection conditions were merged with the discretized data. A white list

indicating the parent-to-child relationship from "genotype" to "ME6" corresponding to the module containing LTβR was included in the Bayesian network learning process. A final network was generated using the bn_tabu_gen function with 500 bootstrap replicates, "bds" score, and iss set to 1. Inverse relationships between nodes were detected using the InverseARCs function from GmicR with default settings.

Immunoblot analysis and antibodies. Tissues were homogenized in phosphate-buffered saline (PBS) containing cOmplete protease inhibitor cocktail (Roche) using the Precellys homogenizer (Bertin). The protein concentration was measured using the Pierce BCA protein assay kit (Thermo Scientific) according to the manufacturer's protocol. Samples (10 μg/lane) were separated by 4 to 12% SDS-PAGE, followed by electrophoretic transfer to nitrocellulose membranes before blocking and incubation with the primary antibodies listed in Table S2. Horseradish peroxidase (HRP)-labeled anti-rabbit or anti-mouse antibodies (Cell Signaling Technologies) were used as secondary antibodies. Relative signal intensity of protein bands was quantified using ImageJ (NIH).

tSNE. The cloud-based platform Cytobank (75) (Mountain View) was used for visualization of flow cytometry data. A total of 60,000 events per sample were analyzed (parameters: iterations, 2,400; perplexity, 80; Theta, 0.5) before overlaid dot plots were generated.

Flow cytometry. Spleens were harvested and digested for 30 min at 37°C using collagenase D (100 mg/ml) and DNase I (20,000 U/ml). Tissue digest was stopped using 1× PBS containing 10 mM EDTA before cell solution was filtered using a 70-μm cell strainer. A red blood cell (RBC) lysis (Merck) was performed before cell numbers were calculated. Single-cell suspended splenocytes (1×10^6 cells) were stained with the fixable viability dye eFluor 780 (eBioscience). Surface staining with antibodies specific for CD3e (145-2c11), CD4 (RM4-5), CD8a (53-6.7), CD19 (6D5), CD25 (3C7), and NK1.1 (PK136), all purchased from BioLegend (expect for CD4, which as purchased from BD Bioscience), was performed. For intracellular staining, splenocytes were incubated for 20 h with toxoplasma lysate antigen (TLA, 15 μg/ml) before brefeldin A (eBioscience) was added for an additional 4 h. After surface staining with anti-CD4 (RM4-5), anti-CD8a (53-6.7), anti-CD107a (1D4B), and anti-TCRb (H57-597), cells were fixed, permeabilized, and stained with anti-IFN-γ (XMG1.2), anti-granzyme B (QA16A02), and antiperforin (S16009A) (all purchased from BioLegend) using a Fix & Perm cell permeabilization kit (Life Technologies) according to the manufacturer's protocol. Major histocompatibility complex class I-SVLAFRRL pentamer was purchased from ProImmune and used in experiments as indicated. BD Calibrate beads (BD Bioscience) were added to the samples before acquisition with a BD LSRFortessa instrument.

Detection of *T. gondii*-specific antibodies. A RecomLine *Toxoplasma* IgG/IgM kit (Mikrogen Diagnostik) was used to detect IgM and IgG antibodies against *T. gondii* in serum. Anti-human IgM and IgG conjugates provided within the kit were replaced with anti-mouse IgM-HRP-labeled (Invitrogen) and anti-mouse IgG-HRP-labeled (Invitrogen) conjugates. Otherwise, the assay was performed according to the manufacturer's protocol.

Serum transfer. Blood from naive donor mice (control serum) or WT mice infected i.p. with 20 cysts of the ME49 strain of *T. gondii* (immune serum) was collected from the vena cava inferior. After 2 h of incubation at room temperature (RT), serum was collected by centrifugation of the blood. Acceptor WT and LTβR$^{-/-}$ mice were reconstituted intraperitoneally with 0.2 ml serum 1 day prior to infection (d-1) as well as on days 3, 7, and 11 p.i. Acceptor (WT and LTβR$^{-/-}$) mice as well as IFN-γ$^{-/-}$ control mice were intraperitoneally infected with 10 cysts (ME49 strain) and weighed and scored daily for the duration of the experiment. *T. gondii*-specific antibodies were detected via line blots to confirm the presence and assess the amount of *T. gondii*-specific antibodies in control and immune serum.

Statistical analysis. Data were analyzed with Prism version 8 (GraphPad) using a log rank (Mantel Cox) test or 2-way analysis of variance (ANOVA) corrected for multiple comparison by the Tukey's or Sidak's *post hoc* test as indicated in the figure legends. Symbols represent individual animals, columns represent mean values, and error bars represent the ± standard error of the mean (SEM). *P* values of ≤0.0332 were considered statistically significant and marked with asterisks. *P* values of ≥0.0332 were considered statistically not significant and were not specifically marked.

Data availability. The data that support the findings of this study are available from the corresponding author.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 6.3 MB.

ACKNOWLEDGMENTS

We thank Nicole Küpper, Julia Mock, and Karin Buchholz for technical assistance. Computational support of the Zentrum für Informations und Medientechnologie, especially the HPC team (High Performance Computing) at the Heinrich Heine University is acknowledged.

This work was supported by the Jürgen Manchot Foundation (Molecules of Infection III [MOI III]). C.F.W. has sponsored research projects with E. Lilly Co. and Capella Biosciences. C.F.W. was supported in part by Perkins Family Foundation and NIH R01AI158552. R.V.-S. was supported by a Perkins Family Foundation fellowship.

A.T. performed and analyzed all experiments, except for Fig. 2a, 3a, 4, and 7a as well as Fig. S2 and S7; R.V.-S. developed the immune network models for analysis of RNA-seq data sets. M.H. performed the experiments illustrated in Fig. 4. P.P. and K.K. performed the RNA sequencing. A.T., U.R.S., and K.P. wrote the manuscript with input from D.D., I.R.D., and C.F.W. K.P., U.R.S. and A.T. designed the study.

We declare no competing interests.

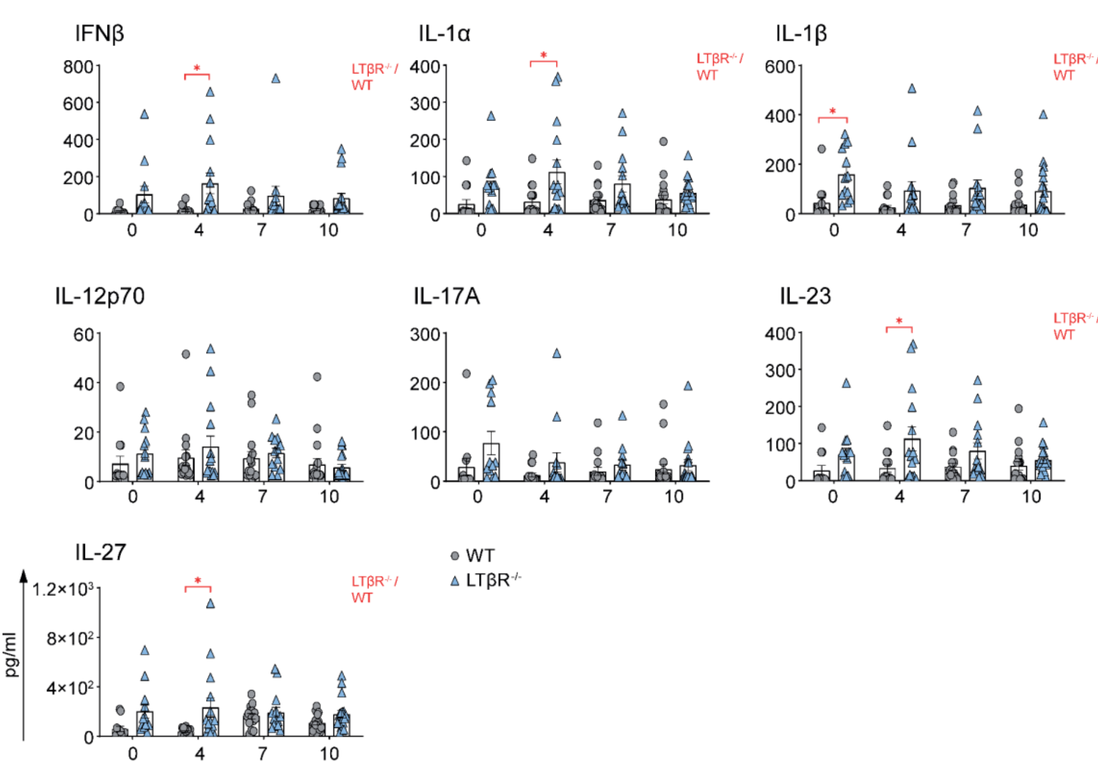
REFERENCES

1. Ward-Kavanagh LK, Lin WW, Sedy JR, Ware CF. 2016. The TNF receptor superfamily in co-stimulating and co-inhibitory responses. Immunity 44:1005–1019. https://doi.org/10.1016/j.immuni.2016.04.019.
2. Hehlgans T, Pfeffer K. 2005. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. Immunology 115:1–20. https://doi.org/10.1111/j.1365-2567.2005.02143.x.
3. Schneider K, Potter KG, Ware CF. 2004. Lymphotoxin and LIGHT signaling pathways and target genes. Immunol Rev 202:49–66. https://doi.org/10.1111/j.0105-2896.2004.00206.x.
4. Ware CF. 2005. Network communications: lymphotoxins, LIGHT, and TNF. Annu Rev Immunol 23:787–819. https://doi.org/10.1146/annurev.immunol.23.021704.115719.
5. Ehlers S, Holscher C, Scheu S, Tertilt C, Hehlgans T, Suwinski J, Endres R, Pfeffer K. 2003. The lymphotoxin beta receptor is critically involved in controlling infections with the intracellular pathogens Mycobacterium tuberculosis and Listeria monocytogenes. J Immunol 170:5210–5218. https://doi.org/10.4049/jimmunol.170.10.5210.
6. Spahn TW, Maaser C, Eckmann L, Heidemann J, Lugering A, Newberry R, Domschke W, Herbst H, Kucharzik T. 2004. The lymphotoxin-beta receptor is critical for control of murine Citrobacter rodentium-induced colitis. Gastroenterology 127:1463–1473. https://doi.org/10.1053/j.gastro.2004.08.022.
7. Futterer A, Mink K, Luz A, Kosco-Vilbois MH, Pfeffer K. 1998. The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. Immunity 9:59–70. https://doi.org/10.1016/s1074-7613(00)80588-9.
8. Wu Q, Sun Y, Wang J, Lin X, Wang Y, Pegg LE, Futterer A, Pfeffer K, Fu YX. 2001. Signal via lymphotoxin-beta R on bone marrow stromal cells is required for an early checkpoint of NK cell development. J Immunol 166:1684–1689. https://doi.org/10.4049/jimmunol.166.3.1684.
9. Banks TA, Rickert S, Ware CF. 2006. Restoring immune defenses via lymphotoxin signaling: lessons from cytomegalovirus. Immunol Res 34:243–254. https://doi.org/10.1385/IR:34:3:243.
10. Puglielli MT, Browning JL, Brewer AW, Schreiber RD, Shieh WJ, Altman JD, Oldstone MB, Zaki SR, Ahmed R. 1999. Reversal of virus-induced systemic shock and respiratory failure by blockade of the lymphotoxin pathway. Nat Med 5:1370–1374. https://doi.org/10.1038/70938.
11. Jin L, Guo X, Shen C, Hao X, Sun P, Li P, Xu T, Hu C, Rose O, Zhou H, Yang M, Qin CF, Guo J, Peng H, Zhu M, Cheng G, Qi X, Lai R. 2018. Salivary factor LTRIN from Aedes aegypti facilitates the transmission of Zika virus by interfering with the lymphotoxin-beta receptor. Nat Immunol 19:342–353. https://doi.org/10.1038/s41590-018-0063-9.
12. Behnke K, Sorg UR, Gabbert HE, Pfeffer K. 2017. The lymphotoxin beta receptor is essential for upregulation of IFN-induced guanylate-binding proteins and survival after Toxoplasma gondii infection. Mediators Inflamm 2017:7375818. https://doi.org/10.1155/2017/7375818.
13. Hill DE, Chirukandoth S, Dubey JP. 2005. Biology and epidemiology of Toxoplasma gondii in man and animals. Anim Health Res Rev 6:41–61. https://doi.org/10.1079/ahr2005100.
14. Saadatnia G, Golkar M. 2012. A review on human toxoplasmosis. Scand J Infect Dis 44:805–814. https://doi.org/10.3109/00365548.2012.693197.
15. Montoya JG, Liesenfeld O. 2004. Toxoplasmosis. Lancet 363:1965–1976. https://doi.org/10.1016/S0140-6736(04)16412-X.
16. Hakimi MA, Olias P, Sibley LD. 2017. Toxoplasma effectors targeting host signaling and transcription. Clin Microbiol Rev 30:615–645. https://doi.org/10.1128/CMR.00005-17.
17. Petersen E. 2007. Toxoplasmosis. Semin Fetal Neonatal Med 12:214–223. https://doi.org/10.1016/j.siny.2007.01.011.
18. Gaddi PJ, Yap GS. 2007. Cytokine regulation of immunopathology in toxoplasmosis. Immunol Cell Biol 85:155–159. https://doi.org/10.1038/sj.icb.7100038.
19. Denkers EY. 1999. T lymphocyte-dependent effector mechanisms of immunity to Toxoplasma gondii. Microbes Infect 1:699–708. https://doi.org/10.1016/S1286-4579(99)80071-9.
20. Ivanova DL, Denton SL, Fettel KD, Sondgeroth KS, Munoz Gutierrez J, Bangoura B, Dunay IR, Gigley JP. 2019. Innate lymphoid cells in protection, pathology, and adaptive immunity during apicomplexan infection. Front Immunol 10:196. https://doi.org/10.3389/fimmu.2019.00196.
21. Darwich L, Coma G, Pena R, Bellido R, Blanco EJ, Este JA, Borras FE, Clotet B, Ruiz L, Rosell A, Andreo F, Parkhouse RM, Bofill M. 2009. Secretion of interferon-gamma by human macrophages demonstrated at the single-cell level after costimulation with interleukin (IL)-12 plus IL-18. Immunology 126:386–393. https://doi.org/10.1111/j.1365-2567.2008.02905.x.
22. Suzuki Y, Orellana MA, Schreiber RD, Remington JS. 1988. Interferon-gamma: the major mediator of resistance against Toxoplasma gondii. Science 240:516–518. https://doi.org/10.1126/science.3128869.
23. Yap GS, Sher A. 1999. Cell-mediated immunity to Toxoplasma gondii: initiation, regulation and effector function. Immunobiology 201:240–247. https://doi.org/10.1016/S0171-2985(99)80064-3.
24. Saeij JP, Frickel EM. 2017. Exposing Toxoplasma gondii hiding inside the vacuole: a role for GBPs, autophagy and host cell death. Curr Opin Microbiol 40:72–80. https://doi.org/10.1016/j.mib.2017.10.021.
25. Schluter D, Kwok LY, Lutjen S, Soltek S, Hoffmann S, Korner H, Deckert M. 2003. Both lymphotoxin-alpha and TNF are crucial for control of Toxoplasma gondii in the central nervous system. J Immunol 170:6172–6182. https://doi.org/10.4049/jimmunol.170.12.6172.
26. Daubener W, Remscheid C, Nockemann S, Pilz K, Seghrouchni S, Mackenzie C, Hadding U. 1996. Anti-parasitic effector mechanisms in human brain tumor cells: role of interferon-gamma and tumor necrosis factor-alpha. Eur J Immunol 26:487–492. https://doi.org/10.1002/eji.1830260231.
27. Fox BA, Gigley JP, Bzik DJ. 2004. Toxoplasma gondii lacks the enzymes required for de novo arginine biosynthesis and arginine starvation triggers cyst formation. Int J Parasitol 34:323–331. https://doi.org/10.1016/j.ijpara.2003.12.001.
28. Pfefferkorn ER, Rebhun S, Eckel M. 1986. Characterization of an indoleamine 2,3-dioxygenase induced by gamma-interferon in cultured human fibroblasts. J Interferon Res 6:267–279. https://doi.org/10.1089/jir.1986.6.267.
29. Scharton-Kersten TM, Yap G, Magram J, Sher A. 1997. Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen Toxoplasma gondii. J Exp Med 185:1261–1273. https://doi.org/10.1084/jem.185.7.1261.
30. Degrandi D, Konermann C, Beuter-Gunia C, Kresse A, Wurthner J, Kurig S, Beer S, Pfeffer K. 2007. Extensive characterization of IFN-induced GTPases mGBP1 to mGBP10 involved in host defense. J Immunol 179:7729–7740. https://doi.org/10.4049/jimmunol.179.11.7729.
31. Steffens N, Beuter-Gunia C, Kravets E, Reich A, Legewie L, Pfeffer K, Degrandi D. 2020. Essential role of mGBP7 for survival of Toxoplasma gondii infection. mBio 11:e02993-19.https://doi.org/10.1128/mBio.02993-19.
32. Yamamoto M, Okuyama M, Ma JS, Kimura T, Kamiyama N, Saiga H, Ohshima J, Sasai M, Kayama H, Okamoto T, Huang DC, Soldati-Favre D, Horie K, Takeda J, Takeda K. 2012. A cluster of interferon-gamma-inducible p65 GTPases plays a critical role in host defense against Toxoplasma gondii. Immunity 37:302–313. https://doi.org/10.1016/j.immuni.2012.06.009.
33. Degrandi D, Kravets E, Konermann C, Beuter-Gunia C, Klumpers V, Lahme S, Wischmann E, Mausberg AK, Beer-Hammer S, Pfeffer K. 2013. Murine guanylate binding protein 2 (mGBP2) controls Toxoplasma gondii replication. Proc Natl Acad Sci U S A 110:294–299. https://doi.org/10.1073/pnas.1205635110.
34. Clough B, Frickel EM. 2017. The toxoplasma parasitophorous vacuole: an evolving host-parasite frontier. Trends Parasitol 33:473–488. https://doi.org/10.1016/j.pt.2017.02.007.

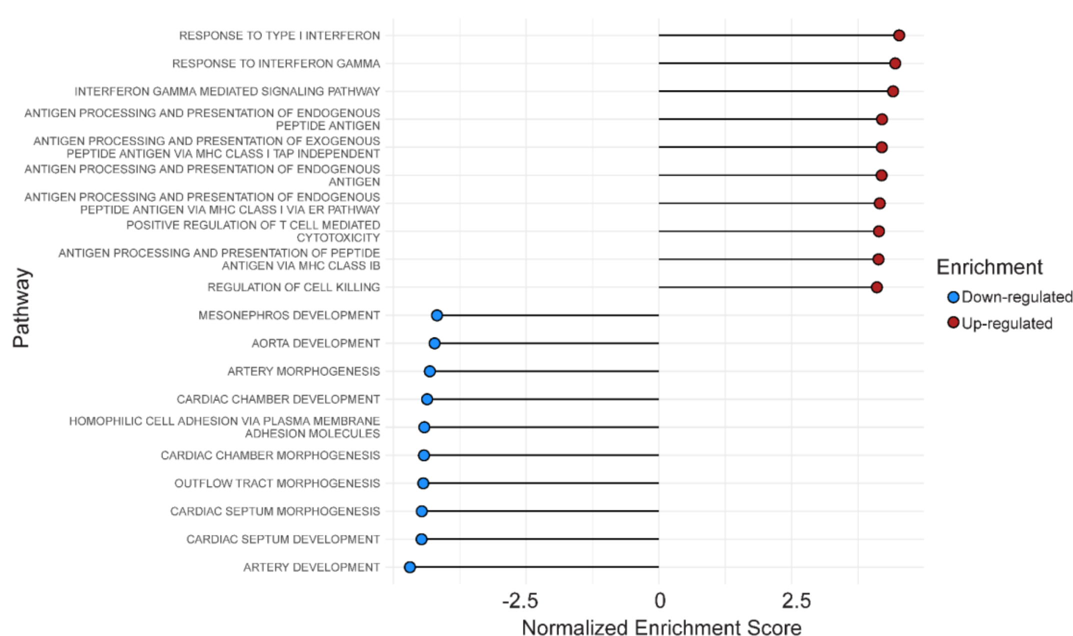
35. Selleck EM, Fentress SJ, Beatty WL, Degrandi D, Pfeffer K, Virgin HW, IV, Macmicking JD, Sibley LD. 2013. Guanylate-binding protein 1 (Gbp1) contributes to cell-autonomous immunity against Toxoplasma gondii. PLoS Pathog 9:e1003320. https://doi.org/10.1371/journal.ppat.1003320.
36. Kravets E, Degrandi D, Ma Q, Peulen TO, Klumpers V, Felekyan S, Kuhnemuth R, Weidtkamp-Peters S, Seidel CA, Pfeffer K. 2016. Guanylate binding proteins directly attack Toxoplasma gondii via supramolecular complexes. Elife 5:e11479. https://doi.org/10.7554/eLife.11479.
37. Yap GS, Scharton-Kersten T, Charest H, Sher A. 1998. Decreased resistance of TNF receptor p55- and p75-deficient mice to chronic toxoplasmosis despite normal activation of inducible nitric oxide synthase in vivo. J Immunology 160:1340–1345.
38. Deckert-Schluter M, Bluethmann H, Rang A, Hof H, Schluter D. 1998. Crucial role of TNF receptor type 1 (p55), but not of TNF receptor type 2 (p75), in murine toxoplasmosis. J Immunology 160:3427–3436.
39. Glatman Zaretsky A, Silver JS, Siwicki M, Durham A, Ware CF, Hunter CA. 2012. Infection with Toxoplasma gondii alters lymphotoxin expression associated with changes in splenic architecture. Infect Immun 80:3602–3610. https://doi.org/10.1128/IAI.00333-12.
40. Gazzinelli RT, Eltoum I, Wynn TA, Sher A. 1993. Acute cerebral toxoplasmosis is induced by in vivo neutralization of TNF-alpha and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation. J Immunol 151:3672–3681.
41. Schaper F, Rose-John S. 2015. Interleukin-6: biology, signaling and strategies of blockade. Cytokine Growth Factor Rev 26:475–487. https://doi.org/10.1016/j.cytogfr.2015.07.004.
42. Saraiva M, O'Garra A. 2010. The regulation of IL-10 production by immune cells. Nat Rev Immunol 10:170–181. https://doi.org/10.1038/nri2711.
43. Brenier-Pinchart MP, Villena I, Mercier C, Durand F, Simon J, Cesbron-Delauw MF, Pelloux H. 2006. The Toxoplasma surface protein SAG1 triggers efficient in vitro secretion of chemokine ligand 2 (CCL2) from human fibroblasts. Microbes Infect 8:254–261. https://doi.org/10.1016/j.micinf.2005.06.023.
44. Singhania A, Graham CM, Gabrysova L, Moreira-Teixeira L, Stavropoulos E, Pitt JM, Chakravarty P, Warnatsch A, Branchett WJ, Conejero L, Lin JW, Davidson S, Wilson MS, Bancroft G, Langhorne J, Frickel E, Sesay AK, Priestnall SL, Herbert E, Ioannou M, Wang Q, Humphreys IR, Dodd J, Openshaw PJM, Mayer-Barber KD, Jankovic D, Sher A, Lloyd CM, Baldwin N, Chaussabel D, Papayannopoulos V, Wack A, Banchereau JF, Pascual VM, O'Garra A. 2019. Transcriptional profiling unveils type I and II interferon networks in blood and tissues across diseases. Nat Commun 10:2887. https://doi.org/10.1038/s41467-019-10601-6.
45. Yarovinsky F. 2014. Innate immunity to Toxoplasma gondii infection. Nat Rev Immunol 14:109–121. https://doi.org/10.1038/nri3598.
46. Ratna A, Arora SK. 2016. Leishmania recombinant antigen modulates macrophage effector function facilitating early clearance of intracellular parasites. Trans R Soc Trop Med Hyg 110:610–619. https://doi.org/10.1093/trstmh/trw068.
47. Koo SJ, Chowdhury IH, Szczesny B, Wan X, Garg NJ. 2016. Macrophages promote oxidative metabolism to drive nitric oxide generation in response to Trypanosoma cruzi. Infect Immun 84:3527–3541. https://doi.org/10.1128/IAI.00809-16.
48. Ufermann CM, Domrose A, Babel T, Tersteegen A, Cengiz SC, Eller SK, Spekker-Bosker K, Sorg UR, Forster I, Daubener W. 2019. Indoleamine 2,3-dioxygenase activity during acute toxoplasmosis and the suppressed T cell proliferation in mice. Front Cell Infect Microbiol 9:184. https://doi.org/10.3389/fcimb.2019.00184.
49. Pittman KJ, Knoll LJ. 2015. Long-Term Relationships: the Complicated Interplay between the Host and the Developmental Stages of Toxoplasma gondii during Acute and Chronic Infections. Microbiol Mol Biol Rev 79:387–401. https://doi.org/10.1128/MMBR.00027-15.
50. Sher A, Denkers EY, Gazzinelli RT. 1995. Induction and regulation of host cell-mediated immunity by Toxoplasma gondii. Ciba Found Symp 195:95–104. discussion 104–9. https://doi.org/10.1002/9780470514849.ch7.
51. Park E, Patel S, Wang Q, Andhey P, Zaitsev K, Porter S, Hershey M, Bern M, Plougastel-Douglas B, Collins P, Colonna M, Murphy KM, Oltz E, Artyomov M, Sibley LD, Yokoyama WM. 2019. Toxoplasma gondii infection drives conversion of NK cells into ILC1-like cells. Elife 8:95. https://doi.org/10.7554/eLife.47605.
52. Vallabhapurapu S, Powolny-Budnicka I, Riemann M, Schmid RM, Paxian S, Pfeffer K, Korner H, Weih F. 2008. Rel/NF-kappaB family member RelA regulates NK1.1- to NK1.1+ transition as well as IL-15-induced expansion of NKT cells. Eur J Immunol 38:3508–3519. https://doi.org/10.1002/eji.200737830.
53. Deckert-Schluter M, Rang A, Weiner D, Huang S, Wiestler OD, Hof H, Schluter D. 1996. Interferon-gamma receptor-deficiency renders mice highly susceptible to toxoplasmosis by decreased macrophage activation. Lab Invest 75:827–841.
54. Zhang Y, Kim TJ, Wroblewska JA, Tesic V, Upadhyay V, Weichselbaum RR, Tumanov AV, Tang H, Guo X, Tang H, Fu YX. 2018. Type 3 innate lymphoid cell-derived lymphotoxin prevents microbiota-dependent inflammation. Cell Mol Immunol 15:697–709. https://doi.org/10.1038/cmi.2017.25.
55. Gazzinelli RT, Wysocka M, Hieny S, Scharton-Kersten T, Cheever A, Kuhn R, Muller W, Trinchieri G, Sher A. 1996. In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. J Immunol 157:798–805.
56. Frey RS, Rahman A, Kefer JC, Minshall RD, Malik AB. 2002. PKCzeta regulates TNF-alpha-induced activation of NADPH oxidase in endothelial cells. Circ Res 90:1012–1019. https://doi.org/10.1161/01.res.0000017631.28815.8e.
57. Ziltener P, Reinheckel T, Oxenius A. 2016. Neutrophil and alveolar macrophage-mediated innate immune control of Legionella pneumophila lung infection via TNF and ROS. PLoS Pathog 12:e1005591. https://doi.org/10.1371/journal.ppat.1005591.
58. Ohshima J, Lee Y, Sasai M, Saitoh T, Su Ma J, Kamiyama N, Matsuura Y, Pann-Ghill S, Hayashi M, Ebisu S, Takeda K, Akira S, Yamamoto M. 2014. Role of mouse and human autophagy proteins in IFN-gamma-induced cell-autonomous responses against Toxoplasma gondii. J Immunol 192:3328–3335. https://doi.org/10.4049/jimmunol.1302822.
59. Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW. 2002. Signaling through the JAK/STAT pathway, recent advances and future challenges. Gene 285:1–24. https://doi.org/10.1016/s0378-1119(02)00398-0.
60. Greenlund AC, Farrar MA, Viviano BL, Schreiber RD. 1994. Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). EMBO J 13:1591–1600. https://doi.org/10.1002/j.1460-2075.1994.tb06422.x.
61. Kutsch S, Degrandi D, Pfeffer K. 2008. Immediate lymphotoxin beta receptor-mediated transcriptional response in host defense against L. monocytogenes. Immunobiology 213:353–366. https://doi.org/10.1016/j.imbio.2007.10.011.
62. Franki AS, Van Beneden K, Dewint P, Hammond KJ, Lambrecht S, Leclercq G, Kronenberg M, Deforce D, Elewaut D. 2006. A unique lymphotoxin {alpha}beta-dependent pathway regulates thymic emigration of V{alpha}14 invariant natural killer T cells. Proc Natl Acad Sci U S A 103:9160–9165. https://doi.org/10.1073/pnas.0508892103.
63. Ronet C, Darche S, Leite de Moraes M, Miyake S, Yamamura T, Louis JA, Kasper LH, Buzoni-Gatel D. 2005. NKT cells are critical for the initiation of an inflammatory bowel response against Toxoplasma gondii. J Immunol 175:899–908. https://doi.org/10.4049/jimmunol.175.2.899.
64. Nakano Y, Hisaeda H, Sakai T, Ishikawa H, Zhang M, Maekawa Y, Zhang T, Takashima M, Nishitani M, Good RA, Himeno K. 2002. Roles of NKT cells in resistance against infection with Toxoplasma gondii and in expression of heat shock protein 65 in the host macrophages. Microbes Infect 4:1–11. https://doi.org/10.1016/s1286-4579(01)01503-9.
65. Paludan SR. 1998. Interleukin-4 and interferon-gamma: the quintessence of a mutual antagonistic relationship. Scand J Immunol 48:459–468. https://doi.org/10.1046/j.1365-3083.1998.00435.x.
66. De Togni P, Goellner J, Ruddle NH, Streeter PR, Fick A, Mariathasan S, Smith SC, Carlson R, Shornick LP, Strauss-Schoenberger J. 1994. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. Science 264:703–707. https://doi.org/10.1126/science.8171322.
67. Sibley LD, Boothroyd JC. 1992. Virulent strains of Toxoplasma gondii comprise a single clonal lineage. Nature 359:82–85. https://doi.org/10.1038/359082a0.
68. Howe DK, Sibley LD. 1995. Toxoplasma gondii comprises three clonal lineages: correlation of parasite genotype with human disease. J Infect Dis 172:1561–1566. https://doi.org/10.1093/infdis/172.6.1561.
69. Howe DK, Honore S, Derouin F, Sibley LD. 1997. Determination of genotypes of Toxoplasma gondii strains isolated from patients with toxoplasmosis. J Clin Microbiol 35:1411–1414. https://doi.org/10.1128/JCM.35.6.1411-1414.1997.
70. Schlüter D, Däubener W, Schares G, Groß U, Pleyer U, Lüder C. 2014. Animals are key to human toxoplasmosis. Int J Med Microbiol 304:917–929. https://doi.org/10.1016/j.ijmm.2014.09.002.
71. Kang H, Remington JS, Suzuki Y. 2000. Decreased resistance of B cell-deficient mice to infection with Toxoplasma gondii despite unimpaired expression of IFN-gamma, TNF-alpha, and inducible nitric oxide synthase. J Immunol 164:2629–2634. https://doi.org/10.4049/jimmunol.164.5.2629.

72. Schluter D, Deckert M, Hof H, Frei K. 2001. Toxoplasma gondii infection of neurons induces neuronal cytokine and chemokine production, but gamma interferon- and tumor necrosis factor-stimulated neurons fail to inhibit the invasion and growth of T. gondii. Infect Immun 69:7889–7893. https://doi.org/10.1128/IAI.69.12.7889-7893.2001.
73. Zhu W, Li J, Pappoe F, Shen J, Yu L. 2019. Strategies developed by Toxoplasma gondii to survive in the host. Front Microbiol 10:899. https://doi.org/10.3389/fmicb.2019.00899.
74. Virgen-Slane R, Correa RG, Ramezani-Rad P, Steen-Fuentes S, Detanico T, DiCandido MJ, Li J, Ware CF. 2020. Cutting edge: the RNA-binding protein Ewing sarcoma is a novel modulator of lymphotoxin beta receptor signaling. J Immunol 204:1085–1090. https://doi.org/10.4049/jimmunol.1901260.
75. Chen TJ, Kotecha N. 2014. Cytobank: providing an analytics platform for community cytometry data analysis and collaboration. Curr Top Microbiol Immunol 377:127–157. https://doi.org/10.1007/82_2014_364.

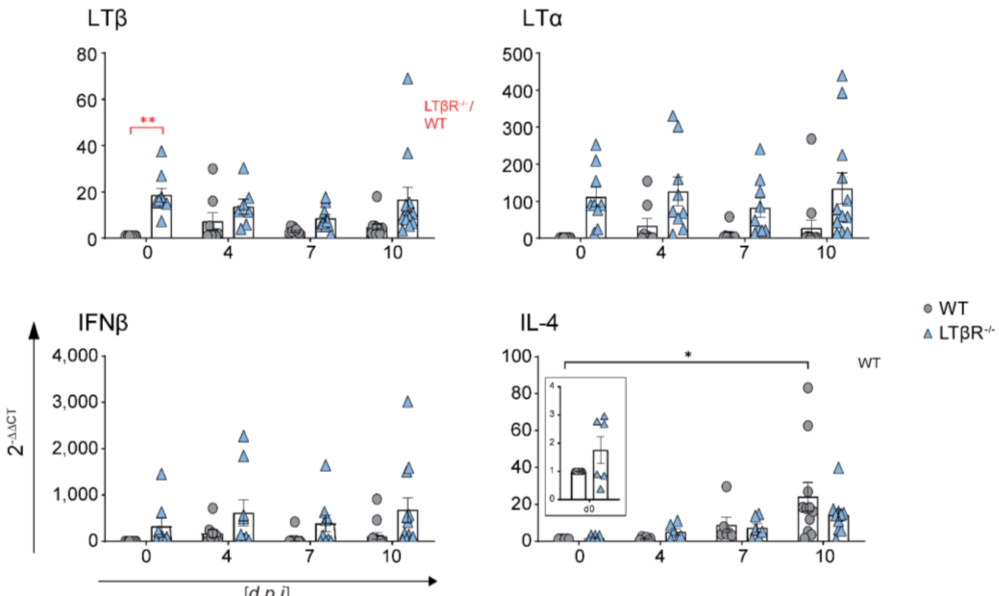
SUPPLEMENTAL MATERIAL



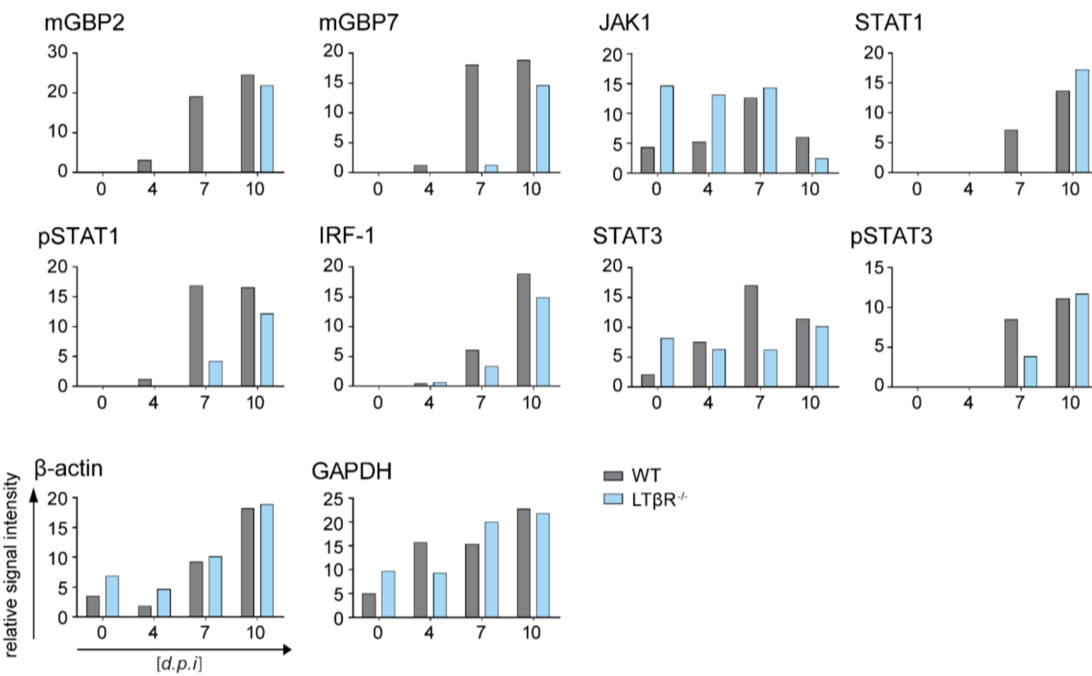
Suppl. Fig. 1 | LTβR$^{-/-}$ mice show dysregulated cytokine expression. Expression of IFNβ, IL-1α, IL-1β, IL-12p70, IL-17A, IL-23 and IL-27 in the serum of uninfected and *T. gondii* infected WT and LTβR$^{-/-}$ mice (d0 - 7: n≥12, d10: n=18) analyzed via bead-based immunoassay. Data shown represent at least three independent experiments; symbols represent individual animals, columns represent mean values and error bars represent ± SEM. 2way ANOVA corrected for multiple comparison by the Tukey's post hoc test was used for statistical analysis. *P<0.0332.



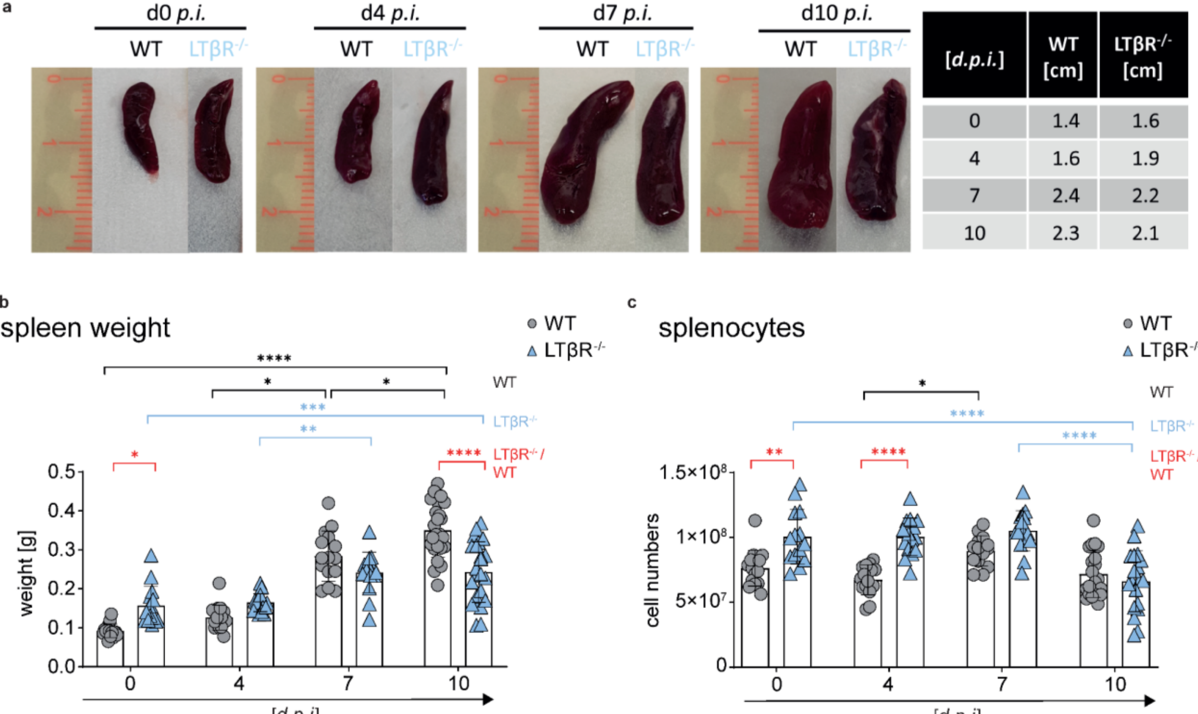
Suppl. Fig. 2 | GSEA. GSEA generated from RNAseq data from lung tissue of *T. gondii* infected (ME49, 40 cysts; d7 *p.i.*) WT mice correlated to infected LTβR$^{-/-}$ mice (n=3/group). GO (Biological Process) molecular signatures were obtained using the msigdr package in R. Gene-level differential expression statistics from infected (d7 *p.i.*) WT mice were tested for geneset enrichment using the fgsea package, with an adjusted p-value of 0.1.



Suppl. Fig. 3 | LTβR$^{-/-}$ mice show dysregulated cytokine expression in lung tissue. qRT-PCR of cytokines (LTβ, LTα, IFNβ, IL-4) in lung tissue from uninfected (d0) and *T. gondii* infected (ME49, 40 cysts *i.p.*) WT and LTβR$^{-/-}$ mice (d0 - 7: n≥12, d10: n≥14; exception: IL-4 n≥6, d0 – 10 *p.i.*). Data shown represent four independent experiments; symbols represent individual animals, columns represent mean values and error bars represent ± SEM. 2way ANOVA corrected for multiple comparison by the Tukey's post hoc test was used for statistical analysis. **P<0.0021.

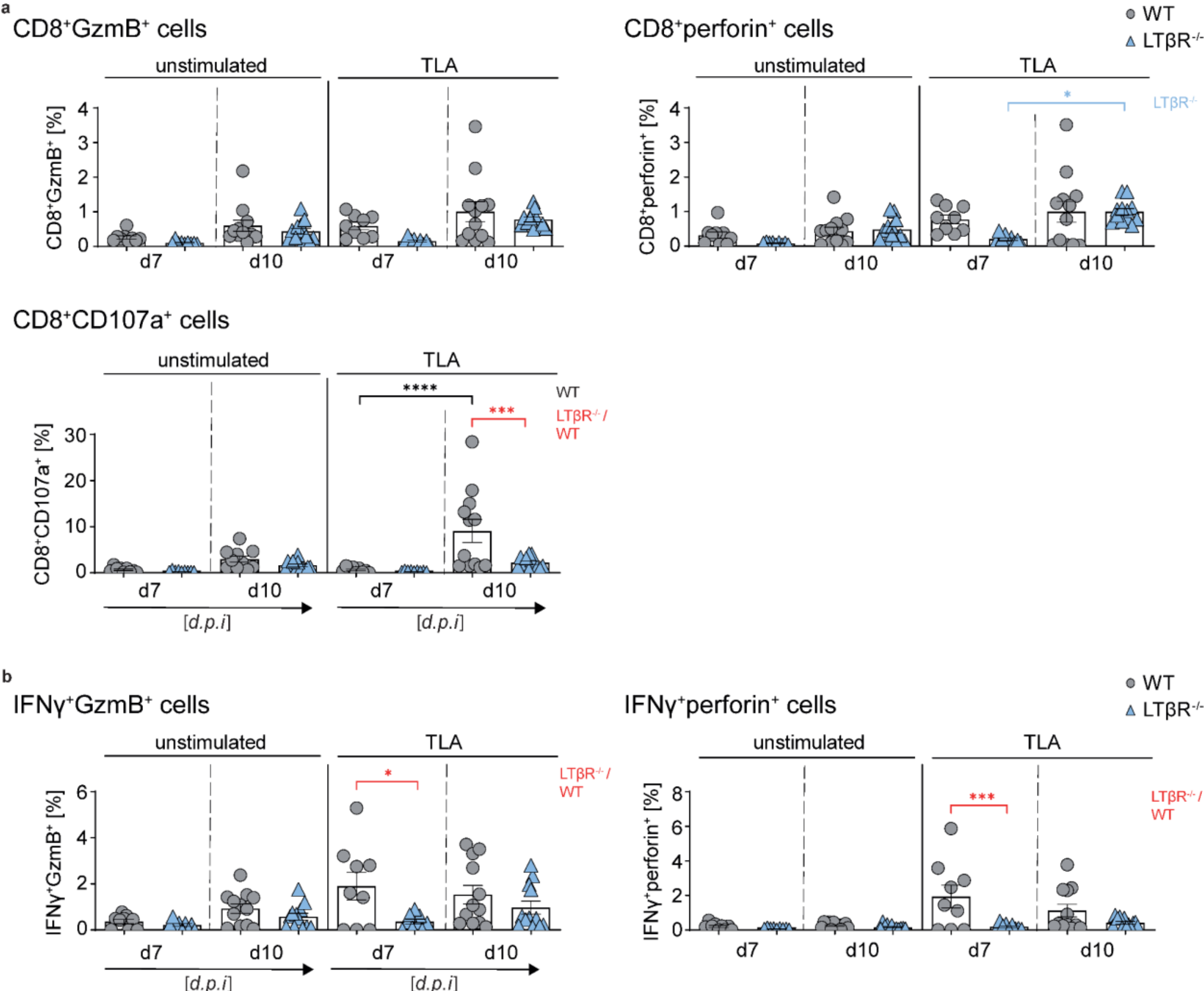


Suppl. Fig. 4 | Immunoblot Quantification. Quantification of relative signal intensities of the immunoblot protein bands shown in Fig. 3c using ImageJ. Data shown are representatives of three independent experiments.

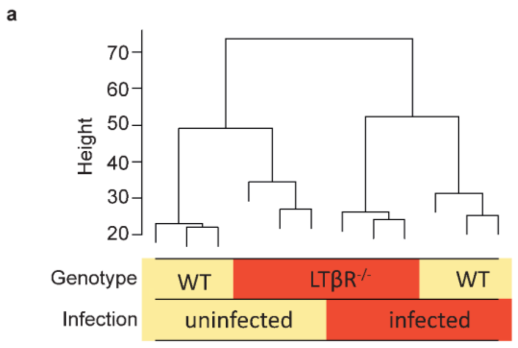


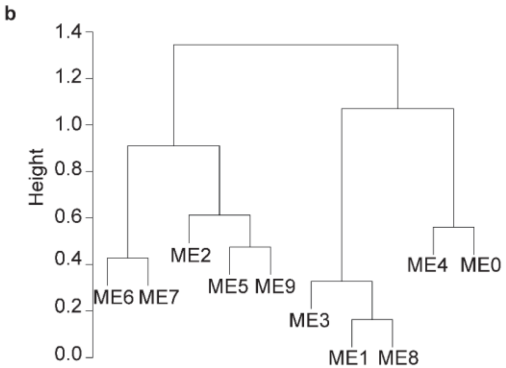
[d.p.i.]	WT [cm]	LTβR-/- [cm]
0	1.4	1.6
4	1.6	1.9
7	2.4	2.2
10	2.3	2.1

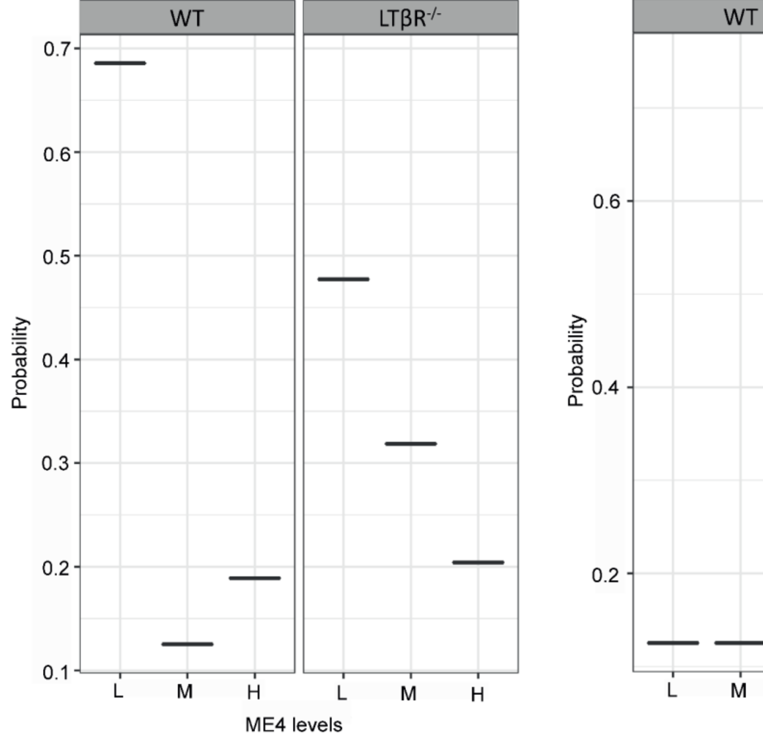
Suppl. Fig. 5 | Decreased spleen weight but increased splenocyte numbers. a, Depiction of representative spleens from uninfected (d0) and *T. gondii* infected (ME49, 40 cysts, *i.p.*) WT and LTβR$^{-/-}$ mice (d0 - 7 *p.i.*: n=9, d10 *p.i.*: n=≥9). Ruler indicated for scale [cm]. **b**, Spleen weight and **c** absolute splenocyte numbers from uninfected and *T. gondii* infected WT and LTβR$^{-/-}$ mice (d0 – 7 *p.i.*: n=15, d10 *p.i.*: n≥19). Data shown in **b** and **c** represent four independent experiments; symbols represent individual animals, columns represent mean values and error bars represent ± SEM. 2way ANOVA corrected for multiple comparison by the Tukey's post hoc test was used for statistical analysis represented in **b** and **c**. *P<0.0332, **P<0.0021, ***P<0.0002 and ****P<0.0001.



Suppl. Fig. 6│LTβR deficiency impairs CD8 T cell effector function in the spleen. Intracellular staining of **a**, cytotoxic granule (GzmB⁺ or perforin⁺) containing and degranulating (CD107a⁺) CD8⁺ T cells and **b**, IFNγ⁺GzmB⁺ and IFNγ⁺perforin⁺ cells [%] of unstimulated and toxoplasma lysate antigen (TLA) *ex vivo* restimulated splenocytes from *T. gondii* infected (d7 and 10 *p.i.*) WT and LTβR$^{-/-}$ mice (d7: n≥6, d10: n≥10). Representative data of at least two independent experiments; symbols represent individual animals, columns represent mean values and error bars represent ± SEM. 2way ANOVA corrected for multiple comparison by the Tukey's post hoc test was used for statistical analysis. *P<0.0332, ***P<0.0002, ****P<0.0001.







Suppl. Fig. 7|Samples, modules and bayesian interference of key relationships from RNAseq analysis. a, Sample dendrogram (hierarchical clustering) with trait heatmap of RNAseq data from lung tissue of uninfected (d0) and *T. gondii* infected (ME49, 40 cysts; d7 *p.i.*) WT and LTβR$^{-/-}$ mice (n=3). Expression data was filtered for differentially expressed genes (all conditions ~10,000 genes). Hierarchical clustering is illustrated as sample dendrogram including a trait heat map (red and yellow bars) for sample classification. For infection, the yellow bar shows clustering of uninfected (d0) and the red bar shows clustering of infected (d7 *p.i.*) animals. For genotype, yellow bars show clustering of WT and the red bar shows clustering of LTβR$^{-/-}$ animals. **b**, For module detection GmicR package in R was used, using a minimum module size of 30, mergeCutHeight of 0.3, and Rsquared cut of 0.80. Gene expression data was condensed into ten module eigengenes (ME0-9) before being used for generation of a host-pathogen network. **c**, The probability of ME4 levels during infection for WT and LTβR$^{-/-}$ mice is shown. ME4 shows a slight increase to the probability of being in a high expression level (represented as "H") in LTβR$^{-/-}$ mice. **d**, The probability of ME3 levels during infection for WT and LTβR$^{-/-}$ mice is shown. ME3 shows reduced probability of being in a high expression level (represented as "H") in LTβR$^{-/-}$ mice. Bayesian inference of the GmicR network was carried out using the bnlearn and gRain packages.

Supplementary Table 1: Primer and probes qRT-PCR.

mGBP1	41	fwd	CAGACTCCTGGAAAGGGACTC
		rev	CTTGGACCTGGAACATTCACTGAC
mGBP2	17	fwd	TGAGTACCTGGAACATTCACTGAC
		rev	AGTCGCGGCTCATTAAAGC
mGBP3	21	fwd	GGCTGAGGACTGTCCCTGT
		rev	CATGGTCCACTCGGAAGC
mGBP5	48	fwd	TCACTGAAGCTGAAGCAAGG
		rev	GCGTCAAAAACAAAGCATTTC
mGBP6/10	6TM*	fwd	ATATTTCAACATTTTTTGTTCCTTGT
		rev	GAAATGGGAGAAAAAATAAATGAAGC
mGBP7	93	fwd	GCAGAGAATCCGGTGCAG
		rev	TTTCCACTAGGCACACAGGA
mGBP8	8TM*	fwd	AAGAAGCTGAAGGAACAAAAGGC
		rev	GAAATGGGAGAAAAAATAAATGAAGC
mGBP9	9TM*	fwd	TTCCAAAACTTTCTCCAGTCACAGTA
		rev	GGCACGCTCCTCTGCAA
IFNγ	63	fwd	ATCTGGAGGAACTGGCAAAA
		rev	TTCAAGACTTCAAAGAGTCTGAGGTA
IFNβ	95	fwd	CAGGCAACCTTTAAGCATCAG
		rev	CCTTTGACCTTTCAAATGCAG
iNOS	13	fwd	CTTTGCCACGGACGAGAC
		rev	TGTACTCTGAGGGCTGACACA
IL-4	2	fwd	CATCGGCATTTTGAACGAG
		rev	CGAGCTCACTCTCTGTGGTG
LTα	62	fwd	TCCCTCAGAAGCACTTGACC
		rev	GAGTTCTGCTTGCTGGGGTA
LTβ	76	fwd	CCTGGTGACCCTGTTGTTG
		rev	TGCTCCTGAGCCAATGATCT
TNFα	49	fwd	TCTTCTCATTCCTGCTTGTGG
		rev	GGTCTGGGCCATAGAACTGA
NOX2-pg91phox	20	fwd	TGCCAACTTCCTCAGCTACA
		rev	GTGCACAGCAAAGTGATTGG
IDO1	2	fwd	GGGCTTCTTCCTCGTCTCTC
		rev	TGGATACAGTGGGGATTGCT
β-actin	106	fwd	TGACAGGATGCAGAAGGAGA
		rev	CGCTCAGGAGGAGCAATG
TgB1	TgB1*	fwd	GCTAAAGGCGTCATTGCTGTT
		rev	GGCGGAACCAACGGAAAT

Primer (Metabion, Martiensried,Germany); numbered probes (Universal ProbeLibrary, Roche, Mannheim, Germany);TM probes (TipMolBIOL, Berlin, Germany); TgB1 probe (Metabion, Martiensried,Germany):

6TM [5' ->3']: FAM-AGT CAT GTT CAA TCT TCT CCC TCT TGT CC-BHQ1
8TM [5' ->3']: FAM-TGT TTC AGT TGC TGT ATC TCT CCG TCC A-BHQ1
9TM [5' ->3']: FAM-CCA GCA GTG AGG GCT CTA TCT GCC T-TMR
TgB1 [5'->3']: FAM-ATC GCA ACG GAG TTC TTC CCA GAC GT-BHQ1

Supplementary Table 2: Antibodies immunoblots.

Primary antibodies	Host species	Company
anti-Jak1 (D1T6W)	mouse	CST
anti-Stat1	rabbit	CST
anti-p-Stat1 (Tyr701)	rabbit	CST
anti-IRF-1 (D5E4) XP®	rabbit	CST
anti-Stat3 (79D7)	rabbit	CST
anti-p-Stat3 (Tyr705) (D3A7) XP®	rabbit	CST
anti-mGBP2	rabbit	Eurogentec
anti-mGBP7	rabbit	Eurogentec
anti-β-Actin (8H10D10)	mouse	CST
anti-GAPDH)14C10)	rabbit	CST

abcam, Cambridge, UK; Cell Signaling Technology (CST), Frankfurt, Germany; Eurogentec, Lüttich, Belgium

4. Discussion and concluding remarks

B cells are an essential part of the immune response against *T. gondii*, as B cell-deficient mice exhibit significantly reduced resistance to the parasite despite maintaining a normal IFNγ production [273-276]. LTβR$^{-/-}$ mice show increased susceptibility to *T. gondii* infection, characterized by impaired innate and adaptive immune responses and increased mortality compared to WT animals [242,248]. LTβR$^{-/-}$ mice exhibit a pronounced deficiency in their humoral immune response, with only low amounts of parasite-specific IgM and no parasite-specific IgG detected in their serum during *T. gondii* infection [248]. This study aimed to further investigate B cell- and antibody-mediated protection and contains two major parts: the investigation of B cell (sub)populations in the BM and PerC of LTβR$^{-/-}$ mice, and the analysis of antibody-mediated protection through passive immunization of LTβR$^{-/-}$ mice during *T. gondii* infection.

Several previously not described alterations were identified in the BM and PerC of LTβR$^{-/-}$. BM B cell frequencies were significantly increased compared to WT mice prior to and during infection, predominantly due to an expansion of mature B cells, whereas earlier developmental stages were mostly similar between genotypes. While developing B cells leave the BM to complete their development in SLOs, they can re-enter the BM as mature B cells, residing in extravascular perisinusoidal niches with BMDCs and T cells, and participating in immune responses against blood-borne pathogens [277-279]. The status of these BMDCs in the LTβR$^{-/-}$ BM is currently unknown but of great interest, as these cells produce MIF, which is a crucial survival factor for mature B cells in the BM [277,278,280,281]. One hypothesis is that the absence of SLOs in LTβR$^{-/-}$ mice [187,234] may promote B cell accumulation in alternative niches, such as the BM or PerC. Alternatively, LTβR signaling might influence B cell migratory properties, in analogy to its established role in thymic T cell migration [249].

The frequency of mature B cells in the BM of LTβR$^{-/-}$ mice did not change during *T. gondii* infection, which is in contrast to the significant reduction found in WT mice. Multiple factors may contribute to this observation. One possibility is that infection-induced changes in the BM microenvironment, which could affect the mature B cells, might occur later LTβR$^{-/-}$ mice. This is supported by the BM GSEA data that indicates reduced or delayed inflammatory responses in LTβR$^{-/-}$ compared to WT BM. And although mature BM B cells are less reliant on CXCL12 for retention compared to earlier developmental stages, they still depend on this chemokine for proper BM localization [35,282,283], which could explain the decrease of mature BM B cells in WT animals. Conversely, the persistence of mature B cells in LTβR$^{-/-}$ BM despite declining CXCL12 levels is remarkable and suggests the involvement of altered or unidentified retention mechanisms, warranting further investigation. Regarding early B cells, both genotypes exhibited significant reductions in these populations in the BM on day 9 p.i., likely due to the inflammation-induced CXCL12 depletion [283]. Unfortunately, these mobilized early B cells were

not detectable in the blood of WT and LTβR$^{-/-}$ mice on day 9 p.i. as they likely underwent apoptosis and were cleared by this time, given their high sensitivity to apoptosis [284]. Thus, future studies should examine this earlier, on days 7 or 8 p.i., to gain better insights into the dynamics of early B cell egress.

The delayed expression of IFNγ and IFNγ-induced effector molecules like mGBPs, IDO or iNOS, which are key players in the cell-autonomous immunity against *T. gondii*, marks a profound disadvantage for LTβR$^{-/-}$ mice after infection [98,99,120,242,248]. BM mRNA sequencing revealed reduced expression of IFN-related gene sets in LTβR$^{-/-}$ BM compared to WT BM, underscoring the impaired IFN response in these mice. Conversely, the 'TNFα signaling *via* NF-κB' gene set was upregulated in LTβR$^{-/-}$ BM, suggesting a compensatory mechanism, as LTβR, TNFR1, and TNFR2 share downstream adaptor molecules and signaling pathways [187,188,194,212]. Furthermore, genes associated with migration and adhesion and known to be inducible via LTβR signaling, such as *Vcam-1*, *Icam-1*, *Sele* (E-selectin), *Cxcl12*, *Cxcl13*, and *MAdCAM-1* [226,238,285-287], were not among the top 40 differentially expressed genes between WT and LTβR$^{-/-}$ BM. However, given the variability in their expression among the biological triplicates (data not shown), future RNA sequencing or gene expression studies with larger sample sizes could provide a more robust analysis of these target genes. Other genes involved in chemotaxis, such as *Ccl6*, *Ccl9*, *Fn1*, *Ccr2* and *Cxcl9,* were identified to be differentially expressed and should be included in future investigations. The LTβR$^{-/-}$ BM also contained increased immunoglobulin transcript levels (*Igkc*, *Ighm*, *Iglc2* and *Ighd*), likely reflecting the higher abundance of mature B cells or an altered B cell activation status. Single cell sequencing could elucidate the impact of absent LTβR signaling on the homeostasis of mature B-2, B-1a and B-1b cells. B-1a cells are of particular interest, as their frequencies were not increased in LTβR$^{-/-}$ mice, unlike other examined B cell subpopulations.

Additionally, single cell RNA sequencing and flow cytometry could be employed to investigate the dynamics of LTβR-ligand expression on B cell subsets and during *T. gondii* infection. For instance, LIGHT, which is absent from naïve and regulatory T cells but present on activated effector and memory T cells and myeloid inflammatory effector cells, is primarily expressed in proinflammatory settings [200,288-290]. Notably, LIGHT is not as commonly associated with B cells as it is with T cells and other immune cell types. Similar to its T cell expression pattern, LIGHT is also virtually absent from naïve B cells but can be induced upon activation, for example *via* CD40 stimulation [200,288,290-292]. On the other hand, surface LT$\alpha_1\beta_2$ is present on naïve B cells in LNs and the spleen where it is upregulated after antigen encounter, but it is significantly reduced on B cells in the blood and BM [288,293]. This pattern highlights the importance of LT$\alpha_1\beta_2$ for B cell functions in the LNs and spleen, including T cell zone and B cell follicle organization, GC formation, and FDC homeostasis [237,271,272,294,295]. It also suggests that LT$\alpha_1\beta_2$ expression is downregulated as B cells migrate out of these compartments [293]. Investigation of the dynamics of LT$\alpha_1\beta_2$ and LIGHT expression on transitional and mature BM B cells, as well as on peritoneal B cell subpopulations, could reveal alterations in LTβR$^{-/-}$ mice that might help

to elucidate the role of LTβR signaling on B cell homeostasis in these compartments. It should also be kept in mind that *T. gondii* infection itself might further modulate $LT\alpha_1\beta_2$ and LIGHT expression on activated B cells.

In addition to B cells, the frequencies of several other immune cell populations were altered in the BM and PerC of $LT\beta R^{-/-}$ mice. $MHCII^+$ monocytes/macrophages were reduced in the BM and spleen of $LT\beta R^{-/-}$ mice, presumably due to the absence of NK cells, which produce IFNγ and prime monocytes during *T. gondii* infection in WT mice [109,296]. This defect in MHCII expression and antigen presentation, along with the absence of NK cells, was corroborated by the BM mRNA analysis, which revealed significantly reduced expression of MHCII- and NK cell-associated genes in $LT\beta R^{-/-}$ mice. Interestingly, a $CD11b^+$ $AA4.1^+$ population was significantly increased in the blood of WT mice compared to $LT\beta R^{-/-}$ mice on day 9 p.i. This population likely consists of peripheral blood monocytes, as these cells also express AA4.1 (also known as CD93 and C1qRp) [297,298], which would add altered monocyte mobilization to the defective activation phenotype observed in the BM of $LT\beta R^{-/-}$ mice.

T cell subpopulations were also affected by the absence of LTβR signaling. In the BM of $LT\beta R^{-/-}$ mice, DN T cells were decreased, while $CD4^+$ and $CD8^+$ T cells frequencies were increased. The ontogeny and functionality of DN T cells remains rather elusive. They can participate in both proinflammatory responses and immune regulation, including the inhibition of B cell proliferation [299,300]. Therefore, it is conceivable that their reduced presence in $LT\beta R^{-/-}$ mice could contribute to the increased B cells and immunopathology observed in these mice. Additionally, the $LT\beta R^{-/-}$ PerC immune cell composition was dominated by neutrophils on day 9 p.i., in contrast to the $CD4^+$ T cell-dominated WT PerC. Further characterization of these $CD4^+$ T cells is essential, as they may include IFNγ-producing Th1 cells or immunosuppressive T_{regs}. A reduction in Th1 cells would imply an absent source of IFNγ, while a reduction in T_{regs}, potentially due to impaired migration [301,302], could exacerbate inflammation and immunopathology in $LT\beta R^{-/-}$ mice [303-306], particularly given the potentially amplified pathogenicity of neutrophils in these mice [193].

While B cells are an essential part of the immune response against *T. gondii*, the extent to which antibodies mediate protection against this parasite in $LT\beta R^{-/-}$ mice was rather unclear, which is why it was further investigated in this study. Both active immunization, which aims to stimulate endogenous antibody production, and passive immunization, where pre-formed exogenous antibodies are transferred to recipients, have been employed in other studies to confer protection against this parasite, although with mixed success. On the one hand, numerous studies demonstrate that antibodies provide a certain degree of protection against *T. gondii*, achieved through both active and passive immunization [274,275,307-317]. Mechanistically, antibodies neutralize and opsonize the parasites, inhibiting their ability to invade host cells and promoting their phagocytosis and destruction

by mononuclear phagocytes [308,311,318-323]. On the other hand, some studies report a lack of significant enhancement of the humoral response in immunized subjects [324-326], and no human vaccine against *T. gondii* is currently accepted, highlighting both the limitations of antibody-mediated protection and the challenges of vaccine development [89,327]. The evaluation of antibody-mediated protection is further complicated by the various inbred mouse strains, parasite strains, routes of infection, and methods of immunization employed by these studies.

In this study, passive immunization prolonged survival but failed to effectively reduce parasite numbers in the lung, spleen, and PerC of LTβR$^{-/-}$ mice. It is possible that the effectiveness of the transferred antibodies is further restricted in LTβR$^{-/-}$ mice due to the absence or impaired function of effector cells that typically target opsonized parasites. For example, NK cells mediate antibody-dependent cellular cytotoxicity (ADCC) [328] and are absent in LTβR$^{-/-}$ mice, while aforementioned monocytes/macrophages show reduced MHCII expression, indicating insufficient priming and probably limited ability to participate in ADCC or parasite phagocytosis in these mice. Furthermore, humoral immunity against *T. gondii* has been shown to depend on help from CD4$^+$ T cells [310], which are functionally impaired in LTβR$^{-/-}$ mice [248] and exhibit altered kinetics in the peritoneal cavity during infection, as demonstrated in this study. It should also be kept in mind that the transferred immune serum likely contained not only antibodies but also other host- and parasite-derived proteins or factors, which could have positively or negatively affected the observed protection.

In addition to the functional deficiencies caused by the absence of LTβR signaling, *T. gondii* further undermines the host's humoral response through immune evasion and manipulation. The parasite can nonspecifically suppress antibody responses through the induction of suppressor macrophages, which inhibit the proliferative responses of nearby target cells through direct cell-to-cell contact [325,329-331]. Intriguingly, parasite-induced suppressor macrophages occur in several inbred mouse strains, including C57BL/6 mice, but are notably absent in BALB/c mice [332]. *T. gondii* possesses an extensive arsenal of both known and yet-to-be-identified molecules capable of manipulating various immune cell populations, including B cells [167,169,185,235,333-335]. Other unicellular parasites also target B cell responses. For example, *Trypanosoma cruzi*, another obligate intracellular parasite, affects both immature and mature B cell subsets. In *T. cruzi*-infected mice, immature B cells in the BM undergo apoptosis, mediated by a soluble product of the cyclooxygenase (Cox) pathway secreted by CD11b$^+$ cells [336]. Additionally, *T. cruzi*-infected mature B cells upregulate Fas and Fas ligand (FasL) on their surface, inducing apoptosis in neighboring B cells, particularly parasite-specific IgG$^+$ B lymphocytes [337]. Furthermore, infections with *T. cruzi* as well as *Plasmodium chabaudi* also lead to a severe reduction in early B cell stages in the BM, attributed to B cell egress triggered by reduced CXCL12 concentrations rather than apoptosis [338,339], similar to our observations during *T. gondii* infection. However, this phenomenon appears to stem from general inflammation rather than a parasite-specific

strategy, as it has also been observed in experiments involving immunization-induced inflammation in the absence of any pathogen [283]. Other parasite-induced mechanisms targeting the B cell response include the activation of polyclonal B cells in the spleen, which inhibits the GC-dependent production of high-affinity, parasite-specific antibodies, as well as the disruption of established memory B cell-mediated protection [340]. Therefore, known (and unknown) counteractive mechanisms employed by *T. gondii* may further diminish the effectiveness of antibody-mediated protection in LTβR$^{-/-}$ mice.

Furthermore, a notable outcome of passive immunization was the specific suppression of endogenously produced antibodies against *T. gondii* in WT recipient mice. We assume that the transferred parasite-specific IgG antibodies are responsible, as this antibody isotype is well-known for its ability to suppress antibody production, with multiple underlying mechanisms being discussed [341]. Optimization of antibody concentration may reduce this effect, as high dosages have been shown to be detrimental compared to lower doses under certain circumstances [342]. LTβR$^{-/-}$ immune serum recipients had low amounts of parasite-specific antibodies in their serum, which presumably originate from the transferred serum rather than endogenous production. In line with this, LTβR$^{-/-}$ control serum recipients did not exhibit any *T. gondii*-specific IgM and IgG on day 9 p.i., underscoring the severe impact of their deficiencies on their humoral response. In contrast, in the study by Tersteegen et al., LTβR$^{-/-}$ mice also completely lacked parasite-specific IgG but displayed low amounts of parasite-specific IgM in their serum on day 10 p.i. [248], suggesting rudimentary production. Due to the dysfunctional GC reaction in LTβR$^{-/-}$ mice, it could be speculated that the parasite-specific IgM in these mice are of low-affinity and could be derived from B-1 cells and B-1-derived PCs, which are major producers of circulating polyreactive natural IgM and low-affinity IgM during infection, independently of GC reactions [42,45,274,343-346]. Regarding the IgM$^+$ rather than IgA$^+$ PCs found in LTβR$^{-/-}$ BM, it is possible that this population may, at least in part, consist of B-1-derived PCs. Additionally, activated BM B-2 cells, which are capable of T cell-independent IgM secretion in response to blood-borne microbes, may contribute to the pool of parasite-specific IgM in LTβR$^{-/-}$ mice [277]. While this would not explain the contradictory data regarding the absence (this study) or presence [248] of parasite-specific IgM in LTβR$^{-/-}$ mice, it nevertheless would be interesting to investigate B-1 cell subsets in the spleen and BM of LTβR$^{-/-}$ mice, as B-1 cells in these compartments rather than those within the peritoneal cavity were identified as major source of natural IgM [346].

More recently, *Nfkbid*, encoding IκBNS, an atypical NF-κB inhibitor with restricted nuclear localization, has been identified as a central regulator of both B-1 and B-2 development and immune responses to *T. gondii* infection [274]. The study by Souza et al. highlights that NF-κB, the major downstream signaling pathway downstream of the LTβR, is critically involved in humoral immunity against *T. gondii*. It would be interesting to explore the expression of *Nfkbid* and other NF-κB-related genes in mature B-1a, B-1b and B-2 cells in LTβR$^{-/-}$ mice, for example, *via* single cell RNA sequencing. While the LTβR is

not expressed on B cells and thus cannot directly affect NF-κB signaling in these cells, its absence may indirectly affect this crucial signaling pathway through altered expression of cytokines, chemokines, or surface signaling molecules on neighboring stromal or myeloid cells.

In summary, the passive immunization protocol in this study could be further optimized, particularly in terms of antibody concentration to minimize the endogenous antibody suppression, and the route and frequency of administration to enhance overall protection. Although the IgG subclass is likely responsible for the immunosuppressive effect observed in this study, excluding IgGs from future approaches would be premature, as they are an important component of the humoral immune response against *T. gondii* [274,308,312,313,320,322].

Finally, the use of mice with targeted LTβR inactivation in DCs (LTβR-CD11cCre) and myeloid cells (LTβR-LysMCre) allows the assessment of cell type-specific roles of LTβR signaling. Although the survival data on these mouse strains have not reached statistical significance so far and require further corroboration, they suggest that LTβR-LysMCre mice exhibit intermediate susceptibility to *T. gondii* infection, positioned between LTβR$^{-/-}$ and WT mice. It would be interesting to assess the impact of myeloid-specific LTβR inactivation on parameters such as splenic architecture, immune cell homeostasis, organ infiltration, parasite-specific antibody production, and to investigate the status of the various B cell subpopulations analyzed in this study. Furthermore, the survival data hint at a possible sexual dimorphism, with male LTβR-LysMCre mice showing increased susceptibility compared to females during acute *T. gondii* infection. While this requires further investigation, it underscores the importance of considering sex as a variable in immunological research, especially in the context of emerging sex-specific treatments in human medicine.

In conclusion, the findings of this study expand our knowledge about the B cell compartment and antibody-mediated protection in LTβR$^{-/-}$ mice during *T. gondii* infection. This includes the BM RNA sequencing results which highlight severely impaired IFN-related responses in LTβR$^{-/-}$ mice, consistent with previous studies [242,248], and provide new directions for future gene expression analysis. Furthermore, while LTβR signaling was previously known to influence T cell migration and development in the thymus [249], lymphocyte homing to LNs [286], the organization of B and T cells in the spleen [187,233,234,243,293,294], and the trafficking of T_{regs} [301,302], the results from this study strongly suggest that LTβR signaling also plays a role in the homeostasis and/or migration of mature B cells. These findings point to new interesting areas of future research for elucidating the role of LTβR signaling in B cell development and could pave the way for the exploration of potential therapeutic strategies targeting toxoplasmosis.

5. References

1. Murphy, K.P., and Weaver, C. (2018). Janeway Immunologie, 9. Auflage Edition (Springer Spektrum).

2. Janeway, C.A., Jr., and Medzhitov, R. (2002). *Innate immune recognition*. Annu Rev Immunol ***20***, 197-216. 10.1146/annurev.immunol.20.083001.084359.

3. Cheng, H., Zheng, Z., and Cheng, T. (2020). *New paradigms on hematopoietic stem cell differentiation*. Protein Cell ***11***, 34-44. 10.1007/s13238-019-0633-0.

4. Dress, R.J., Dutertre, C.A., Giladi, A., Schlitzer, A., Low, I., Shadan, N.B., Tay, A., Lum, J., Kairi, M., Hwang, Y.Y., Becht, E., Cheng, Y., Chevrier, M., Larbi, A., Newell, E.W., Amit, I., Chen, J., and Ginhoux, F. (2019). *Plasmacytoid dendritic cells develop from Ly6D(+) lymphoid progenitors distinct from the myeloid lineage*. Nat Immunol ***20***, 852-864. 10.1038/s41590-019-0420-3.

5. Sun, L., Su, Y., Jiao, A., Wang, X., and Zhang, B. (2023). *T cells in health and disease*. Signal Transduct Target Ther ***8***, 235. 10.1038/s41392-023-01471-y.

6. Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L., and Wiley, D.C. (1987). *The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens*. Nature ***329***, 512-518. 10.1038/329512a0.

7. Townsend, A., and Bodmer, H. (1989). *Antigen recognition by class I-restricted T lymphocytes*. Annu Rev Immunol ***7***, 601-624. 10.1146/annurev.iy.07.040189.003125.

8. Crotty, S. (2015). *A brief history of T cell help to B cells*. Nat Rev Immunol ***15***, 185-189. 10.1038/nri3803.

9. Palma, J., Tokarz-Deptula, B., Deptula, J., and Deptula, W. (2018). *Natural antibodies - facts known and unknown*. Cent Eur J Immunol ***43***, 466-475. 10.5114/ceji.2018.81354.

10. Holodick, N.E., Rodriguez-Zhurbenko, N., and Hernandez, A.M. (2017). *Defining Natural Antibodies*. Front Immunol ***8***, 872. 10.3389/fimmu.2017.00872.

11. Berthelot, J.M., Jamin, C., Amrouche, K., Le Goff, B., Maugars, Y., and Youinou, P. (2013). *Regulatory B cells play a key role in immune system balance*. Joint Bone Spine ***80***, 18-22. 10.1016/j.jbspin.2012.04.010.

12. Adolfsson, J., Mansson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C.T., Bryder, D., Yang, L., Borge, O.J., Thoren, L.A., Anderson, K., Sitnicka, E., Sasaki, Y., Sigvardsson, M., and Jacobsen, S.E. (2005). *Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment*. Cell ***121***, 295-306. 10.1016/j.cell.2005.02.013.

13. Yang, L., Bryder, D., Adolfsson, J., Nygren, J., Mansson, R., Sigvardsson, M., and Jacobsen, S.E. (2005). *Identification of Lin(-)Sca1(+)kit(+)CD34(+)Flt3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients*. Blood ***105***, 2717-2723. 10.1182/blood-2004-06-2159.

14. Luc, S., Buza-Vidas, N., and Jacobsen, S.E. (2008). *Delineating the cellular pathways of hematopoietic lineage commitment*. Semin Immunol ***20***, 213-220. 10.1016/j.smim.2008.07.005.

15. Swiecki, M., and Colonna, M. (2015). *The multifaceted biology of plasmacytoid dendritic cells*. Nat Rev Immunol ***15***, 471-485. 10.1038/nri3865.

16. Sichien, D., Lambrecht, B.N., Guilliams, M., and Scott, C.L. (2017). *Development of conventional dendritic cells: from common bone marrow progenitors to multiple subsets in peripheral tissues*. Mucosal Immunol ***10***, 831-844. 10.1038/mi.2017.8.

17. Musumeci, A., Lutz, K., Winheim, E., and Krug, A.B. (2019). *What Makes a pDC: Recent Advances in Understanding Plasmacytoid DC Development and Heterogeneity*. Front Immunol ***10***, 1222. 10.3389/fimmu.2019.01222.

18. Zehentmeier, S., and Pereira, J.P. (2019). *Cell circuits and niches controlling B cell development*. Immunol Rev ***289***, 142-157. 10.1111/imr.12749.

19. Hardy, R.R., and Hayakawa, K. (2001). *B cell development pathways*. Annu Rev Immunol ***19***, 595-621. 10.1146/annurev.immunol.19.1.595.

20. LeBien, T.W., and Tedder, T.F. (2008). *B lymphocytes: how they develop and function*. Blood ***112***, 1570-1580. 10.1182/blood-2008-02-078071.

21. Schatz, D.G., Oettinger, M.A., and Baltimore, D. (1989). *The V(D)J recombination activating gene, RAG-1*. Cell ***59***, 1035-1048. 10.1016/0092-8674(89)90760-5.

22. Oettinger, M.A., Schatz, D.G., Gorka, C., and Baltimore, D. (1990). *RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination*. Science ***248***, 1517-1523. 10.1126/science.2360047.

23. Sakaguchi, N., and Melchers, F. (1986). *Lambda 5, a new light-chain-related locus selectively expressed in pre-B lymphocytes*. Nature ***324***, 579-582. 10.1038/324579a0.

24. Kudo, A., and Melchers, F. (1987). *A second gene, VpreB in the lambda 5 locus of the mouse, which appears to be selectively expressed in pre-B lymphocytes*. EMBO J ***6***, 2267-2272. 10.1002/j.1460-2075.1987.tb02500.x.

25. Bauer, S.R., Kudo, A., and Melchers, F. (1988). *Structure and pre-B lymphocyte restricted expression of the VpreB in humans and conservation of its structure in other mammalian species*. EMBO J ***7***, 111-116. 10.1002/j.1460-2075.1988.tb02789.x.

26. Karasuyama, H., Kudo, A., and Melchers, F. (1990). *The proteins encoded by the VpreB and lambda 5 pre-B cell-specific genes can associate with each other and with mu heavy chain*. J Exp Med ***172***, 969-972. 10.1084/jem.172.3.969.

27. Ehlich, A., Schaal, S., Gu, H., Kitamura, D., Muller, W., and Rajewsky, K. (1993). *Immunoglobulin heavy and light chain genes rearrange independently at early stages of B cell development*. Cell ***72***, 695-704. 10.1016/0092-8674(93)90398-a.

28. Reth, M.G., Ammirati, P., Jackson, S., and Alt, F.W. (1985). *Regulated progression of a cultured pre-B-cell line to the B-cell stage*. Nature ***317***, 353-355. 10.1038/317353a0.

29. Winkler, T.H., and Martensson, I.L. (2018). *The Role of the Pre-B Cell Receptor in B Cell Development, Repertoire Selection, and Tolerance*. Front Immunol ***9***, 2423. 10.3389/fimmu.2018.02423.

30. Melchers, F. (2015). *Checkpoints that control B cell development*. J Clin Invest ***125***, 2203-2210. 10.1172/JCI78083.

31. Chung, J.B., Silverman, M., and Monroe, J.G. (2003). *Transitional B cells: step by step towards immune competence*. Trends Immunol ***24***, 343-349. 10.1016/s1471-4906(03)00119-4.

32. Enders, A., Short, A., Miosge, L.A., Bergmann, H., Sontani, Y., Bertram, E.M., Whittle, B., Balakishnan, B., Yoshida, K., Sjollema, G., Field, M.A., Andrews, T.D., Hagiwara, H., and Goodnow, C.C. (2014). *Zinc-finger protein ZFP318 is essential for expression of IgD, the alternatively spliced Igh product made by mature B lymphocytes*. Proc Natl Acad Sci U S A ***111***, 4513-4518. 10.1073/pnas.1402739111.

33. Tokoyoda, K., Egawa, T., Sugiyama, T., Choi, B.I., and Nagasawa, T. (2004). *Cellular niches controlling B lymphocyte behavior within bone marrow during development*. Immunity ***20***, 707-718. 10.1016/j.immuni.2004.05.001.

34. Giorgiutti, S., Rottura, J., Korganow, A.S., and Gies, V. (2024). *CXCR4: from B-cell development to B cell-mediated diseases*. Life Sci Alliance ***7***. 10.26508/lsa.202302465.

35. Glodek, A.M., Honczarenko, M., Le, Y., Campbell, J.J., and Silberstein, L.E. (2003). *Sustained activation of cell adhesion is a differentially regulated process in B lymphopoiesis*. J Exp Med ***197***, 461-473. 10.1084/jem.20021477.

36. Beck, T.C., Gomes, A.C., Cyster, J.G., and Pereira, J.P. (2014). *CXCR4 and a cell-extrinsic mechanism control immature B lymphocyte egress from bone marrow*. J Exp Med ***211***, 2567-2581. 10.1084/jem.20140457.

37. Allende, M.L., Tuymetova, G., Lee, B.G., Bonifacino, E., Wu, Y.P., and Proia, R.L. (2010). *S1P1 receptor directs the release of immature B cells from bone marrow into blood*. J Exp Med ***207***, 1113-1124. 10.1084/jem.20092210.

38. Simmons, S., and Ishii, M. (2014). *Sphingosine-1-phosphate: a master regulator of lymphocyte egress and immunity*. Arch Immunol Ther Exp (Warsz) ***62***, 103-115. 10.1007/s00005-013-0264-8.

39. Rolink, A.G., Andersson, J., and Melchers, F. (1998). *Characterization of immature B cells by a novel monoclonal antibody, by turnover and by mitogen reactivity*. Eur J Immunol ***28***, 3738-3748. 10.1002/(SICI)1521-4141(199811)28:11<3738::AID-IMMU3738>3.0.CO;2-Q.

40. Loder, F., Mutschler, B., Ray, R.J., Paige, C.J., Sideras, P., Torres, R., Lamers, M.C., and Carsetti, R. (1999). *B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals*. J Exp Med ***190***, 75-89. 10.1084/jem.190.1.75.

41. Yam-Puc, J.C., Zhang, L., Zhang, Y., and Toellner, K.M. (2018). *Role of B-cell receptors for B-cell development and antigen-induced differentiation*. F1000Res ***7***, 429. 10.12688/f1000research.13567.1.

42. Martin, F., Oliver, A.M., and Kearney, J.F. (2001). *Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens*. Immunity ***14***, 617-629. 10.1016/s1074-7613(01)00129-7.

43. Cerutti, A., Cols, M., and Puga, I. (2013). *Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes*. Nat Rev Immunol ***13***, 118-132. 10.1038/nri3383.

44. Baumgarth, N. (2011). *The double life of a B-1 cell: self-reactivity selects for protective effector functions*. Nat Rev Immunol ***11***, 34-46. 10.1038/nri2901.

45. Baumgarth, N. (2016). *B-1 Cell Heterogeneity and the Regulation of Natural and Antigen-Induced IgM Production*. Front Immunol ***7***, 324. 10.3389/fimmu.2016.00324.

46. Baumgarth, N. (2017). *A Hard(y) Look at B-1 Cell Development and Function*. J Immunol ***199***, 3387-3394. 10.4049/jimmunol.1700943.

47. de Andres, B., Gonzalo, P., Minguet, S., Martinez-Marin, J.A., Soro, P.G., Marcos, M.A., and Gaspar, M.L. (2002). *The first 3 days of B-cell development in the mouse embryo*. Blood ***100***, 4074-4081. 10.1182/blood-2002-03-0809.

48. Esplin, B.L., Welner, R.S., Zhang, Q., Borghesi, L.A., and Kincade, P.W. (2009). *A differentiation pathway for B1 cells in adult bone marrow*. Proc Natl Acad Sci U S A ***106***, 5773-5778. 10.1073/pnas.0811632106.

49. Ghosn, E.E., Sadate-Ngatchou, P., Yang, Y., Herzenberg, L.A., and Herzenberg, L.A. (2011). *Distinct progenitors for B-1 and B-2 cells are present in adult mouse spleen*. Proc Natl Acad Sci U S A ***108***, 2879-2884. 10.1073/pnas.1019764108.

50. Hardy, R.R., and Hayakawa, K. (1991). *A developmental switch in B lymphopoiesis*. Proc Natl Acad Sci U S A ***88***, 11550-11554. 10.1073/pnas.88.24.11550.

51. Hayakawa, K., Hardy, R.R., Herzenberg, L.A., and Herzenberg, L.A. (1985). *Progenitors for Ly-1 B cells are distinct from progenitors for other B cells*. J Exp Med ***161***, 1554-1568. 10.1084/jem.161.6.1554.

52. Montecino-Rodriguez, E., Leathers, H., and Dorshkind, K. (2006). *Identification of a B-1 B cell-specified progenitor*. Nat Immunol ***7***, 293-301. 10.1038/ni1301.

53. Tung, J.W., Mrazek, M.D., Yang, Y., Herzenberg, L.A., and Herzenberg, L.A. (2006). *Phenotypically distinct B cell development pathways map to the three B cell lineages in the mouse*. Proc Natl Acad Sci U S A ***103***, 6293-6298. 10.1073/pnas.0511305103.

54. Haughton, G., Arnold, L.W., Whitmore, A.C., and Clarke, S.H. (1993). *B-1 cells are made, not born*. Immunol Today ***14***, 84-87; discussion 87-91. 10.1016/0167-5699(93)90064-R.

55. Arnold, L.W., Pennell, C.A., McCray, S.K., and Clarke, S.H. (1994). *Development of B-1 cells: segregation of phosphatidyl choline-specific B cells to the B-1 population occurs after immunoglobulin gene expression*. J Exp Med ***179***, 1585-1595. 10.1084/jem.179.5.1585.

56. Wortis, H.H., Teutsch, M., Higer, M., Zheng, J., and Parker, D.C. (1995). *B-cell activation by crosslinking of surface IgM or ligation of CD40 involves alternative signal pathways and results in different B-cell phenotypes*. Proc Natl Acad Sci U S A ***92***, 3348-3352. 10.1073/pnas.92.8.3348.

57. Hayakawa, K., Asano, M., Shinton, S.A., Gui, M., Allman, D., Stewart, C.L., Silver, J., and Hardy, R.R. (1999). *Positive selection of natural autoreactive B cells*. Science ***285***, 113-116. 10.1126/science.285.5424.113.

58. Berland, R., and Wortis, H.H. (2002). *Origins and functions of B-1 cells with notes on the role of CD5*. Annu Rev Immunol ***20***, 253-300. 10.1146/annurev.immunol.20.100301.064833.

59. Graf, R., Seagal, J., Otipoby, K.L., Lam, K.P., Ayoub, S., Zhang, B., Sander, S., Chu, V.T., and Rajewsky, K. (2019). *BCR-dependent lineage plasticity in mature B cells*. Science ***363***, 748-753. 10.1126/science.aau8475.

60. Wong, J.B., Hewitt, S.L., Heltemes-Harris, L.M., Mandal, M., Johnson, K., Rajewsky, K., Koralov, S.B., Clark, M.R., Farrar, M.A., and Skok, J.A. (2019). *B-1a cells acquire their unique characteristics by bypassing the pre-BCR selection stage*. Nat Commun ***10***, 4768. 10.1038/s41467-019-12824-z.

61. Hayakawa, K., Hardy, R.R., Parks, D.R., and Herzenberg, L.A. (1983). *The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice*. J Exp Med ***157***, 202-218. 10.1084/jem.157.1.202.

62. Kantor, A.B., Merrill, C.E., Herzenberg, L.A., and Hillson, J.L. (1997). *An unbiased analysis of V(H)-D-J(H) sequences from B-1a, B-1b, and conventional B cells*. J Immunol ***158***, 1175-1186.

63. Duan, B., and Morel, L. (2006). *Role of B-1a cells in autoimmunity*. Autoimmun Rev ***5***, 403-408. 10.1016/j.autrev.2005.10.007.

64. Alugupalli, K.R., Leong, J.M., Woodland, R.T., Muramatsu, M., Honjo, T., and Gerstein, R.M. (2004). *B1b lymphocytes confer T cell-independent long-lasting immunity*. Immunity ***21***, 379-390. 10.1016/j.immuni.2004.06.019.

65. Haas, K.M., Poe, J.C., Steeber, D.A., and Tedder, T.F. (2005). *B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae*. Immunity ***23***, 7-18. 10.1016/j.immuni.2005.04.011.

66. Smith, F.L., and Baumgarth, N. (2019). *B-1 cell responses to infections*. Curr Opin Immunol ***57***, 23-31. 10.1016/j.coi.2018.12.001.

67. Lanzavecchia, A., and Sallusto, F. (2007). *Toll-like receptors and innate immunity in B-cell activation and antibody responses*. Curr Opin Immunol ***19***, 268-274. 10.1016/j.coi.2007.04.002.

68. Cerutti, A., Puga, I., and Cols, M. (2011). *Innate control of B cell responses*. Trends Immunol ***32***, 202-211. 10.1016/j.it.2011.02.004.

69. Mebius, R.E., and Kraal, G. (2005). *Structure and function of the spleen*. Nat Rev Immunol ***5***, 606-616. 10.1038/nri1669.

70. Okada, T., Miller, M.J., Parker, I., Krummel, M.F., Neighbors, M., Hartley, S.B., O'Garra, A., Cahalan, M.D., and Cyster, J.G. (2005). *Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells*. PLoS Biol ***3***, e150. 10.1371/journal.pbio.0030150.

71. MacLennan, I.C., Toellner, K.M., Cunningham, A.F., Serre, K., Sze, D.M., Zuniga, E., Cook, M.C., and Vinuesa, C.G. (2003). *Extrafollicular antibody responses*. Immunol Rev ***194***, 8-18. 10.1034/j.1600-065x.2003.00058.x.

72. Paus, D., Phan, T.G., Chan, T.D., Gardam, S., Basten, A., and Brink, R. (2006). *Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation*. J Exp Med ***203***, 1081-1091. 10.1084/jem.20060087.

73. Nutt, S.L., Hodgkin, P.D., Tarlinton, D.M., and Corcoran, L.M. (2015). *The generation of antibody-secreting plasma cells*. Nat Rev Immunol ***15***, 160-171. 10.1038/nri3795.

74. De Silva, N.S., and Klein, U. (2015). *Dynamics of B cells in germinal centres*. Nat Rev Immunol ***15***, 137-148. 10.1038/nri3804.

75. Victora, G.D., and Nussenzweig, M.C. (2022). *Germinal Centers*. Annu Rev Immunol ***40***, 413-442. 10.1146/annurev-immunol-120419-022408.

76. Chi, X., Li, Y., and Qiu, X. (2020). *V(D)J recombination, somatic hypermutation and class switch recombination of immunoglobulins: mechanism and regulation*. Immunology ***160***, 233-247. 10.1111/imm.13176.

77. Victora, G.D., Schwickert, T.A., Fooksman, D.R., Kamphorst, A.O., Meyer-Hermann, M., Dustin, M.L., and Nussenzweig, M.C. (2010). *Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter*. Cell ***143***, 592-605. 10.1016/j.cell.2010.10.032.

78. Gitlin, A.D., Shulman, Z., and Nussenzweig, M.C. (2014). *Clonal selection in the germinal centre by regulated proliferation and hypermutation*. Nature ***509***, 637-640. 10.1038/nature13300.

79. Kepler, T.B., and Perelson, A.S. (1993). *Cyclic re-entry of germinal center B cells and the efficiency of affinity maturation*. Immunol Today ***14***, 412-415. 10.1016/0167-5699(93)90145-B.

80. Roco, J.A., Mesin, L., Binder, S.C., Nefzger, C., Gonzalez-Figueroa, P., Canete, P.F., Ellyard, J., Shen, Q., Robert, P.A., Cappello, J., Vohra, H., Zhang, Y., Nowosad, C.R., Schiepers, A., Corcoran, L.M., Toellner, K.M., Polo, J.M., Meyer-Hermann, M., Victora, G.D., and Vinuesa, C.G. (2019). *Class-Switch Recombination Occurs Infrequently in Germinal Centers*. Immunity ***51***, 337-350 e337. 10.1016/j.immuni.2019.07.001.

81. Desmonts, G., and Couvreur, J. (1974). *Congenital toxoplasmosis. A prospective study of 378 pregnancies*. N Engl J Med ***290***, 1110-1116. 10.1056/NEJM197405162902003.

82. Dubey, J.P., Cerqueira-Cezar, C.K., Murata, F.H.A., Verma, S.K., Kwok, O.C.H., Pedersen, K., Rosenthal, B.M., and Su, C. (2020). *White-tailed deer (Odocoileus virginianus) are a reservoir of a diversity of Toxoplasma gondii strains in the USA and pose a risk to consumers of undercooked venison*. Parasitology ***147***, 775-781. 10.1017/S0031182020000451.

83. Flegr, J., Prandota, J., Sovickova, M., and Israili, Z.H. (2014). *Toxoplasmosis--a global threat. Correlation of latent toxoplasmosis with specific disease burden in a set of 88 countries*. PLoS One ***9***, e90203. 10.1371/journal.pone.0090203.

84. Galal, L., Hamidovic, A., Darde, M.L., and Mercier, M. (2019). *Diversity of Toxoplasma gondii strains at the global level and its determinants*. Food Waterborne Parasitol ***15***, e00052. 10.1016/j.fawpar.2019.e00052.

85. Howe, D.K., and Sibley, L.D. (1995). *Toxoplasma gondii comprises three clonal lineages: correlation of parasite genotype with human disease*. J Infect Dis ***172***, 1561-1566. 10.1093/infdis/172.6.1561.

86. C, S.S., and Hubal, A. (2023). *Animal Models for Toxoplasma gondii Infection*. Curr Protoc ***3***, e871. 10.1002/cpz1.871.

87. Grigg, M.E., and Boothroyd, J.C. (2001). *Rapid identification of virulent type I strains of the protozoan pathogen Toxoplasma gondii by PCR-restriction fragment length polymorphism analysis at the B1 gene*. J Clin Microbiol ***39***, 398-400. 10.1128/JCM.39.1.398-400.2001.

88. Khan, A., Su, C., German, M., Storch, G.A., Clifford, D.B., and Sibley, L.D. (2005). *Genotyping of Toxoplasma gondii strains from immunocompromised patients reveals high prevalence of type I strains*. J Clin Microbiol ***43***, 5881-5887. 10.1128/JCM.43.12.5881-5887.2005.

89. Zhang, Y., Li, D., Lu, S., and Zheng, B. (2022). *Toxoplasmosis vaccines: what we have and where to go?* NPJ Vaccines ***7***, 131. 10.1038/s41541-022-00563-0.

90. Robert-Gangneux, F., and Darde, M.L. (2012). *Epidemiology of and diagnostic strategies for toxoplasmosis*. Clin Microbiol Rev ***25***, 264-296. 10.1128/CMR.05013-11.

91. Attias, M., Teixeira, D.E., Benchimol, M., Vommaro, R.C., Crepaldi, P.H., and De Souza, W. (2020). *The life-cycle of Toxoplasma gondii reviewed using animations*. Parasit Vectors ***13***, 588. 10.1186/s13071-020-04445-z.

92. Martorelli Di Genova, B., Wilson, S.K., Dubey, J.P., and Knoll, L.J. (2019). *Intestinal delta-6-desaturase activity determines host range for Toxoplasma sexual reproduction*. PLoS Biol ***17***, e3000364. 10.1371/journal.pbio.3000364.

93. Shapiro, K., Bahia-Oliveira, L., Dixon, B., Dumetre, A., de Wit, L.A., VanWormer, E., and Villena, I. (2019). *Environmental transmission of Toxoplasma gondii: Oocysts in water, soil and food*. Food Waterborne Parasitol ***15***, e00049. 10.1016/j.fawpar.2019.e00049.

94. Freppel, W., Ferguson, D.J.P., Shapiro, K., Dubey, J.P., Puech, P.H., and Dumetre, A. (2019). *Structure, composition, and roles of the Toxoplasma gondii oocyst and sporocyst walls*. Cell Surf ***5***, 100016. 10.1016/j.tcsw.2018.100016.

95. Clough, B., and Frickel, E.M. (2017). *The Toxoplasma Parasitophorous Vacuole: An Evolving Host-Parasite Frontier*. Trends Parasitol ***33***, 473-488. 10.1016/j.pt.2017.02.007.

96. Pereira, K.S., Franco, R.M., and Leal, D.A. (2010). *Transmission of toxoplasmosis (Toxoplasma gondii) by foods*. Adv Food Nutr Res ***60***, 1-19. 10.1016/S1043-4526(10)60001-0.

97. Kieffer, F., and Wallon, M. (2013). *Congenital toxoplasmosis*. Handb Clin Neurol ***112***, 1099-1101. 10.1016/B978-0-444-52910-7.00028-3.

98. Yarovinsky, F. (2014). *Innate immunity to Toxoplasma gondii infection*. Nat Rev Immunol ***14***, 109-121. 10.1038/nri3598.

99. Sasai, M., and Yamamoto, M. (2019). *Innate, adaptive, and cell-autonomous immunity against Toxoplasma gondii infection*. Exp Mol Med ***51***, 1-10. 10.1038/s12276-019-0353-9.

100. Yarovinsky, F., Zhang, D., Andersen, J.F., Bannenberg, G.L., Serhan, C.N., Hayden, M.S., Hieny, S., Sutterwala, F.S., Flavell, R.A., Ghosh, S., and Sher, A. (2005). *TLR11 activation of dendritic cells by a protozoan profilin-like protein*. Science ***308***, 1626-1629. 10.1126/science.1109893.

101. Raetz, M., Kibardin, A., Sturge, C.R., Pifer, R., Li, H., Burstein, E., Ozato, K., Larin, S., and Yarovinsky, F. (2013). *Cooperation of TLR12 and TLR11 in the IRF8-dependent IL-12 response to Toxoplasma gondii profilin*. J Immunol ***191***, 4818-4827. 10.4049/jimmunol.1301301.

102. Gazzinelli, R.T., Mendonca-Neto, R., Lilue, J., Howard, J., and Sher, A. (2014). *Innate resistance against Toxoplasma gondii: an evolutionary tale of mice, cats, and men*. Cell Host Microbe ***15***, 132-138. 10.1016/j.chom.2014.01.004.

103. Andrade, W.A., Souza Mdo, C., Ramos-Martinez, E., Nagpal, K., Dutra, M.S., Melo, M.B., Bartholomeu, D.C., Ghosh, S., Golenbock, D.T., and Gazzinelli, R.T. (2013). *Combined action of nucleic acid-sensing Toll-like receptors and TLR11/TLR12 heterodimers imparts resistance to Toxoplasma gondii in mice*. Cell Host Microbe ***13***, 42-53. 10.1016/j.chom.2012.12.003.

104. Aliberti, J., Reis e Sousa, C., Schito, M., Hieny, S., Wells, T., Huffnagle, G.B., and Sher, A. (2000). *CCR5 provides a signal for microbial induced production of IL-12 by CD8 alpha+ dendritic cells*. Nat Immunol ***1***, 83-87. 10.1038/76957.

105. Ibrahim, H.M., Bannai, H., Xuan, X., and Nishikawa, Y. (2009). *Toxoplasma gondii cyclophilin 18-mediated production of nitric oxide induces Bradyzoite conversion in a CCR5-dependent manner*. Infect Immun ***77***, 3686-3695. 10.1128/IAI.00361-09.

106. Nishimura, M., Umeda, K., Suwa, M., Furuoka, H., and Nishikawa, Y. (2017). *CCR5 Is Involved in Interruption of Pregnancy in Mice Infected with Toxoplasma gondii during Early Pregnancy*. Infect Immun ***85***. 10.1128/IAI.00257-17.

107. Gazzinelli, R.T., Hieny, S., Wynn, T.A., Wolf, S., and Sher, A. (1993). *Interleukin 12 is required for the T-lymphocyte-independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts*. Proc Natl Acad Sci U S A ***90***, 6115-6119. 10.1073/pnas.90.13.6115.

108. Hunter, C.A., Subauste, C.S., Van Cleave, V.H., and Remington, J.S. (1994). *Production of gamma interferon by natural killer cells from Toxoplasma gondii-infected SCID mice: regulation by interleukin-10, interleukin-12, and tumor necrosis factor alpha*. Infect Immun ***62***, 2818-2824. 10.1128/iai.62.7.2818-2824.1994.

109. Askenase, M.H., Han, S.J., Byrd, A.L., Morais da Fonseca, D., Bouladoux, N., Wilhelm, C., Konkel, J.E., Hand, T.W., Lacerda-Queiroz, N., Su, X.Z., Trinchieri, G., Grainger, J.R., and Belkaid, Y. (2015). *Bone-Marrow-Resident NK Cells Prime Monocytes for Regulatory Function during Infection*. Immunity ***42***, 1130-1142. 10.1016/j.immuni.2015.05.011.

110. Spits, H., Bernink, J.H., and Lanier, L. (2016). *NK cells and type 1 innate lymphoid cells: partners in host defense*. Nat Immunol ***17***, 758-764. 10.1038/ni.3482.

111. Lopez-Yglesias, A.H., Burger, E., Camanzo, E., Martin, A.T., Araujo, A.M., Kwok, S.F., and Yarovinsky, F. (2021). *T-bet-dependent ILC1- and NK cell-derived IFN-gamma mediates cDC1-dependent host resistance against Toxoplasma gondii*. PLoS Pathog ***17***, e1008299. 10.1371/journal.ppat.1008299.

112. Manetti, R., Parronchi, P., Giudizi, M.G., Piccinni, M.P., Maggi, E., Trinchieri, G., and Romagnani, S. (1993). *Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells*. J Exp Med ***177***, 1199-1204. 10.1084/jem.177.4.1199.

113. Scharton-Kersten, T., Nakajima, H., Yap, G., Sher, A., and Leonard, W.J. (1998). *Infection of mice lacking the common cytokine receptor gamma-chain (gamma(c)) reveals an unexpected role for CD4+ T lymphocytes in early IFN-gamma-dependent resistance to Toxoplasma gondii*. J Immunol ***160***, 2565-2569.

114. Nishiyama, S., Pradipta, A., Ma, J.S., Sasai, M., and Yamamoto, M. (2020). *T cell-derived interferon-gamma is required for host defense to Toxoplasma gondii*. Parasitol Int ***75***, 102049. 10.1016/j.parint.2019.102049.

115. Gazzinelli, R.T., Hakim, F.T., Hieny, S., Shearer, G.M., and Sher, A. (1991). *Synergistic role of CD4+ and CD8+ T lymphocytes in IFN-gamma production and protective immunity induced by an attenuated Toxoplasma gondii vaccine*. J Immunol ***146***, 286-292.

116. Wang, X., Kang, H., Kikuchi, T., and Suzuki, Y. (2004). *Gamma interferon production, but not perforin-mediated cytolytic activity, of T cells is required for prevention of toxoplasmic encephalitis in BALB/c mice genetically resistant to the disease*. Infect Immun ***72***, 4432-4438. 10.1128/IAI.72.8.4432-4438.2004.

117. Suzuki, Y., Claflin, J., Wang, X., Lengi, A., and Kikuchi, T. (2005). *Microglia and macrophages as innate producers of interferon-gamma in the brain following infection with Toxoplasma gondii*. Int J Parasitol ***35***, 83-90. 10.1016/j.ijpara.2004.10.020.

118. Sturge, C.R., Benson, A., Raetz, M., Wilhelm, C.L., Mirpuri, J., Vitetta, E.S., and Yarovinsky, F. (2013). *TLR-independent neutrophil-derived IFN-gamma is important for host resistance to intracellular pathogens*. Proc Natl Acad Sci U S A ***110***, 10711-10716. 10.1073/pnas.1307868110.

119. Biswas, A., French, T., Dusedau, H.P., Mueller, N., Riek-Burchardt, M., Dudeck, A., Bank, U., Schuler, T., and Dunay, I.R. (2017). *Behavior of Neutrophil Granulocytes during Toxoplasma gondii Infection in the Central Nervous System*. Front Cell Infect Microbiol ***7***, 259. 10.3389/fcimb.2017.00259.

120. Suzuki, Y., Orellana, M.A., Schreiber, R.D., and Remington, J.S. (1988). *Interferon-gamma: the major mediator of resistance against Toxoplasma gondii*. Science ***240***, 516-518. 10.1126/science.3128869.

121. Negishi, H., Taniguchi, T., and Yanai, H. (2018). *The Interferon (IFN) Class of Cytokines and the IFN Regulatory Factor (IRF) Transcription Factor Family*. Cold Spring Harb Perspect Biol ***10***. 10.1101/cshperspect.a028423.

122. Platanias, L.C. (2005). *Mechanisms of type-I- and type-II-interferon-mediated signalling*. Nat Rev Immunol ***5***, 375-386. 10.1038/nri1604.

123. Hu, X., Li, J., Fu, M., Zhao, X., and Wang, W. (2021). *The JAK/STAT signaling pathway: from bench to clinic*. Signal Transduct Target Ther ***6***, 402. 10.1038/s41392-021-00791-1.

124. McNab, F., Mayer-Barber, K., Sher, A., Wack, A., and O'Garra, A. (2015). *Type I interferons in infectious disease*. Nat Rev Immunol ***15***, 87-103. 10.1038/nri3787.

125. Levy, D.E., Lew, D.J., Decker, T., Kessler, D.S., and Darnell, J.E., Jr. (1990). *Synergistic interaction between interferon-alpha and interferon-gamma through induced synthesis of one subunit of the transcription factor ISGF3*. EMBO J ***9***, 1105-1111. 10.1002/j.1460-2075.1990.tb08216.x.

126. MacMicking, J., Xie, Q.W., and Nathan, C. (1997). *Nitric oxide and macrophage function*. Annu Rev Immunol ***15***, 323-350. 10.1146/annurev.immunol.15.1.323.

127. Wang, F., Zhang, S., Jeon, R., Vuckovic, I., Jiang, X., Lerman, A., Folmes, C.D., Dzeja, P.D., and Herrmann, J. (2018). *Interferon Gamma Induces Reversible Metabolic Reprogramming of M1 Macrophages to Sustain Cell Viability and Pro-Inflammatory Activity*. EBioMedicine ***30***, 303-316. 10.1016/j.ebiom.2018.02.009.

128. Lazear, H.M., Schoggins, J.W., and Diamond, M.S. (2019). *Shared and Distinct Functions of Type I and Type III Interferons*. Immunity ***50***, 907-923. 10.1016/j.immuni.2019.03.025.

129. Wells, A.I., and Coyne, C.B. (2018). *Type III Interferons in Antiviral Defenses at Barrier Surfaces*. Trends Immunol ***39***, 848-858. 10.1016/j.it.2018.08.008.

130. Lee, A.J., and Ashkar, A.A. (2018). *The Dual Nature of Type I and Type II Interferons*. Front Immunol ***9***, 2061. 10.3389/fimmu.2018.02061.

131. Bekpen, C., Hunn, J.P., Rohde, C., Parvanova, I., Guethlein, L., Dunn, D.M., Glowalla, E., Leptin, M., and Howard, J.C. (2005). *The interferon-inducible p47 (IRG) GTPases in vertebrates: loss of the cell autonomous resistance mechanism in the human lineage*. Genome Biol ***6***, R92. 10.1186/gb-2005-6-11-r92.

132. Degrandi, D., Konermann, C., Beuter-Gunia, C., Kresse, A., Wurthner, J., Kurig, S., Beer, S., and Pfeffer, K. (2007). *Extensive characterization of IFN-induced GTPases mGBP1 to mGBP10 involved in host defense*. J Immunol ***179***, 7729-7740. 10.4049/jimmunol.179.11.7729.

133. Kresse, A., Konermann, C., Degrandi, D., Beuter-Gunia, C., Wuerthner, J., Pfeffer, K., and Beer, S. (2008). *Analyses of murine GBP homology clusters based on in silico, in vitro and in vivo studies*. BMC Genomics ***9***, 158. 10.1186/1471-2164-9-158.

134. Olszewski, M.A., Gray, J., and Vestal, D.J. (2006). *In silico genomic analysis of the human and murine guanylate-binding protein (GBP) gene clusters*. J Interferon Cytokine Res ***26***, 328-352. 10.1089/jir.2006.26.328.

135. Martens, S., Parvanova, I., Zerrahn, J., Griffiths, G., Schell, G., Reichmann, G., and Howard, J.C. (2005). *Disruption of Toxoplasma gondii parasitophorous vacuoles by the mouse p47-resistance GTPases*. PLoS Pathog ***1***, e24. 10.1371/journal.ppat.0010024.

136. Kravets, E., Degrandi, D., Weidtkamp-Peters, S., Ries, B., Konermann, C., Felekyan, S., Dargazanli, J.M., Praefcke, G.J., Seidel, C.A., Schmitt, L., Smits, S.H., and Pfeffer, K. (2012). *The GTPase activity of murine guanylate-binding protein 2 (mGBP2) controls the intracellular localization and recruitment to the parasitophorous vacuole of Toxoplasma gondii*. J Biol Chem ***287***, 27452-27466. 10.1074/jbc.M112.379636.

137. Degrandi, D., Kravets, E., Konermann, C., Beuter-Gunia, C., Klumpers, V., Lahme, S., Rasch, E., Mausberg, A.K., Beer-Hammer, S., and Pfeffer, K. (2013). *Murine guanylate binding protein 2 (mGBP2) controls Toxoplasma gondii replication*. Proc Natl Acad Sci U S A ***110***, 294-299. 10.1073/pnas.1205635110.

138. Selleck, E.M., Fentress, S.J., Beatty, W.L., Degrandi, D., Pfeffer, K., Virgin, H.W.t., Macmicking, J.D., and Sibley, L.D. (2013). *Guanylate-binding protein 1 (Gbp1) contributes to cell-autonomous immunity against Toxoplasma gondii*. PLoS Pathog ***9***, e1003320. 10.1371/journal.ppat.1003320.

139. Taylor, G.A., Collazo, C.M., Yap, G.S., Nguyen, K., Gregorio, T.A., Taylor, L.S., Eagleson, B., Secrest, L., Southon, E.A., Reid, S.W., Tessarollo, L., Bray, M., McVicar, D.W., Komschlies, K.L., Young, H.A., Biron, C.A., Sher, A., and Vande Woude, G.F. (2000). *Pathogen-specific loss of host resistance in mice lacking the IFN-gamma-inducible gene IGTP*. Proc Natl Acad Sci U S A ***97***, 751-755. 10.1073/pnas.97.2.751.

140. Collazo, C.M., Yap, G.S., Sempowski, G.D., Lusby, K.C., Tessarollo, L., Vande Woude, G.F., Sher, A., and Taylor, G.A. (2001). *Inactivation of LRG-47 and IRG-47 reveals a family of interferon gamma-inducible genes with essential, pathogen-specific roles in resistance to infection*. J Exp Med ***194***, 181-188. 10.1084/jem.194.2.181.

141. Collazo, C.M., Yap, G.S., Hieny, S., Caspar, P., Feng, C.G., Taylor, G.A., and Sher, A. (2002). *The function of gamma interferon-inducible GTP-binding protein IGTP in host resistance to Toxoplasma gondii is Stat1 dependent and requires expression in both hematopoietic and nonhematopoietic cellular compartments*. Infect Immun ***70***, 6933-6939. 10.1128/IAI.70.12.6933-6939.2002.

142. Yamamoto, M., Okuyama, M., Ma, J.S., Kimura, T., Kamiyama, N., Saiga, H., Ohshima, J., Sasai, M., Kayama, H., Okamoto, T., Huang, D.C., Soldati-Favre, D., Horie, K., Takeda, J., and Takeda,

K. (2012). *A cluster of interferon-gamma-inducible p65 GTPases plays a critical role in host defense against Toxoplasma gondii*. Immunity ***37***, 302-313. 10.1016/j.immuni.2012.06.009.

143. Steffens, N., Beuter-Gunia, C., Kravets, E., Reich, A., Legewie, L., Pfeffer, K., and Degrandi, D. (2020). *Essential Role of mGBP7 for Survival of Toxoplasma gondii Infection*. mBio ***11***. 10.1128/mBio.02993-19.

144. Pfefferkorn, E.R. (1984). *Interferon gamma blocks the growth of Toxoplasma gondii in human fibroblasts by inducing the host cells to degrade tryptophan*. Proc Natl Acad Sci U S A ***81***, 908-912. 10.1073/pnas.81.3.908.

145. Pfefferkorn, E.R., Rebhun, S., and Eckel, M. (1986). *Characterization of an indoleamine 2,3-dioxygenase induced by gamma-interferon in cultured human fibroblasts*. J Interferon Res ***6***, 267-279. 10.1089/jir.1986.6.267.

146. Butcher, B.A., Fox, B.A., Rommereim, L.M., Kim, S.G., Maurer, K.J., Yarovinsky, F., Herbert, D.R., Bzik, D.J., and Denkers, E.Y. (2011). *Toxoplasma gondii rhoptry kinase ROP16 activates STAT3 and STAT6 resulting in cytokine inhibition and arginase-1-dependent growth control*. PLoS Pathog ***7***, e1002236. 10.1371/journal.ppat.1002236.

147. El Kasmi, K.C., Qualls, J.E., Pesce, J.T., Smith, A.M., Thompson, R.W., Henao-Tamayo, M., Basaraba, R.J., Konig, T., Schleicher, U., Koo, M.S., Kaplan, G., Fitzgerald, K.A., Tuomanen, E.I., Orme, I.M., Kanneganti, T.D., Bogdan, C., Wynn, T.A., and Murray, P.J. (2008). *Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens*. Nat Immunol ***9***, 1399-1406. 10.1038/ni.1671.

148. Fox, B.A., Gigley, J.P., and Bzik, D.J. (2004). *Toxoplasma gondii lacks the enzymes required for de novo arginine biosynthesis and arginine starvation triggers cyst formation*. Int J Parasitol ***34***, 323-331. 10.1016/j.ijpara.2003.12.001.

149. Schluter, D., Deckert-Schluter, M., Lorenz, E., Meyer, T., Rollinghoff, M., and Bogdan, C. (1999). *Inhibition of inducible nitric oxide synthase exacerbates chronic cerebral toxoplasmosis in Toxoplasma gondii-susceptible C57BL/6 mice but does not reactivate the latent disease in T. gondii-resistant BALB/c mice*. J Immunol ***162***, 3512-3518.

150. Divanovic, S., Sawtell, N.M., Trompette, A., Warning, J.I., Dias, A., Cooper, A.M., Yap, G.S., Arditi, M., Shimada, K., Duhadaway, J.B., Prendergast, G.C., Basaraba, R.J., Mellor, A.L., Munn, D.H., Aliberti, J., and Karp, C.L. (2012). *Opposing biological functions of tryptophan catabolizing enzymes during intracellular infection*. J Infect Dis ***205***, 152-161. 10.1093/infdis/jir621.

151. Murakami, Y., Hoshi, M., Hara, A., Takemura, M., Arioka, Y., Yamamoto, Y., Matsunami, H., Funato, T., Seishima, M., and Saito, K. (2012). *Inhibition of increased indoleamine 2,3-dioxygenase activity attenuates Toxoplasma gondii replication in the lung during acute infection*. Cytokine ***59***, 245-251. 10.1016/j.cyto.2012.04.022.

152. Ufermann, C.M., Domrose, A., Babel, T., Tersteegen, A., Cengiz, S.C., Eller, S.K., Spekker-Bosker, K., Sorg, U.R., Forster, I., and Daubener, W. (2019). *Indoleamine 2,3-Dioxygenase Activity During Acute Toxoplasmosis and the Suppressed T Cell Proliferation in Mice*. Front Cell Infect Microbiol ***9***, 184. 10.3389/fcimb.2019.00184.

153. Adams, L.B., Hibbs, J.B., Jr., Taintor, R.R., and Krahenbuhl, J.L. (1990). *Microbiostatic effect of murine-activated macrophages for Toxoplasma gondii. Role for synthesis of inorganic nitrogen oxides from L-arginine*. J Immunol ***144***, 2725-2729.

154. Fang, F.C. (2004). *Antimicrobial reactive oxygen and nitrogen species: concepts and controversies*. Nat Rev Microbiol ***2***, 820-832. 10.1038/nrmicro1004.

155. Zhao, X.Y., Lempke, S.L., Urban Arroyo, J.C., Brown, I.G., Yin, B., Magaj, M.M., Holness, N.K., Smiley, J., Redemann, S., and Ewald, S.E. (2024). *iNOS is necessary for GBP-mediated T. gondii*

clearance in murine macrophages via vacuole nitration and intravacuolar network collapse. Nat Commun **15**, 2698. 10.1038/s41467-024-46790-y.

156. Dincel, G.C., and Atmaca, H.T. (2015). *Nitric oxide production increases during Toxoplasma gondii encephalitis in mice*. Exp Parasitol **156**, 104-112. 10.1016/j.exppara.2015.06.009.

157. Scharton-Kersten, T.M., Yap, G., Magram, J., and Sher, A. (1997). *Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen Toxoplasma gondii*. J Exp Med **185**, 1261-1273. 10.1084/jem.185.7.1261.

158. Kim, J.H., Lee, J., Bae, S.J., Kim, Y., Park, B.J., Choi, J.W., Kwon, J., Cha, G.H., Yoo, H.J., Jo, E.K., Bae, Y.S., Lee, Y.H., and Yuk, J.M. (2017). *NADPH oxidase 4 is required for the generation of macrophage migration inhibitory factor and host defense against Toxoplasma gondii infection*. Sci Rep **7**, 6361. 10.1038/s41598-017-06610-4.

159. Murray, H.W. (1983). *Macrophage oxygen-dependent killing of intracellular parasites: Toxoplasma and Leishmania*. Adv Exp Med Biol **162**, 127-143. 10.1007/978-1-4684-4481-0_13.

160. Murray, H.W., Juangbhanich, C.W., Nathan, C.F., and Cohn, Z.A. (1979). *Macrophage oxygen-dependent antimicrobial activity. II. The role of oxygen intermediates*. J Exp Med **150**, 950-964. 10.1084/jem.150.4.950.

161. Shrestha, S.P., Tomita, T., Weiss, L.M., and Orlofsky, A. (2006). *Proliferation of Toxoplasma gondii in inflammatory macrophages in vivo is associated with diminished oxygen radical production in the host cell*. Int J Parasitol **36**, 433-441. 10.1016/j.ijpara.2006.01.006.

162. Zhuang, H., Yao, C., Zhao, X., Chen, X., Yang, Y., Huang, S., Pan, L., Du, A., and Yang, Y. (2020). *DNA double-strand breaks in the Toxoplasma gondii-infected cells by the action of reactive oxygen species*. Parasit Vectors **13**, 490. 10.1186/s13071-020-04324-7.

163. Szewczyk-Golec, K., Pawlowska, M., Wesolowski, R., Wroblewski, M., and Mila-Kierzenkowska, C. (2021). *Oxidative Stress as a Possible Target in the Treatment of Toxoplasmosis: Perspectives and Ambiguities*. Int J Mol Sci **22**. 10.3390/ijms22115705.

164. Quan, J.H., Gao, F.F., Ma, T.Z., Ye, W., Gao, X., Deng, M.Z., Yin, L.L., Choi, I.W., Yuk, J.M., Cha, G.H., Lee, Y.H., and Chu, J.Q. (2023). *Toxoplasma gondii Induces Pyroptosis in Human Placental Trophoblast and Amniotic Cells by Inducing ROS Production and Activation of Cathepsin B and NLRP1/NLRP3/NLRC4/AIM2 Inflammasome*. Am J Pathol **193**, 2047-2065. 10.1016/j.ajpath.2023.08.016.

165. Pawlowska, M., Mila-Kierzenkowska, C., Szczegielniak, J., and Wozniak, A. (2023). *Oxidative Stress in Parasitic Diseases-Reactive Oxygen Species as Mediators of Interactions between the Host and the Parasites*. Antioxidants (Basel) **13**. 10.3390/antiox13010038.

166. Rosowski, E.E., Nguyen, Q.P., Camejo, A., Spooner, E., and Saeij, J.P. (2014). *Toxoplasma gondii Inhibits gamma interferon (IFN-gamma)- and IFN-beta-induced host cell STAT1 transcriptional activity by increasing the association of STAT1 with DNA*. Infect Immun **82**, 706-719. 10.1128/IAI.01291-13.

167. Olias, P., Etheridge, R.D., Zhang, Y., Holtzman, M.J., and Sibley, L.D. (2016). *Toxoplasma Effector Recruits the Mi-2/NuRD Complex to Repress STAT1 Transcription and Block IFN-gamma-Dependent Gene Expression*. Cell Host Microbe **20**, 72-82. 10.1016/j.chom.2016.06.006.

168. Gay, G., Braun, L., Brenier-Pinchart, M.P., Vollaire, J., Josserand, V., Bertini, R.L., Varesano, A., Touquet, B., De Bock, P.J., Coute, Y., Tardieux, I., Bougdour, A., and Hakimi, M.A. (2016). *Toxoplasma gondii TgIST co-opts host chromatin repressors dampening STAT1-dependent gene regulation and IFN-gamma-mediated host defenses*. J Exp Med **213**, 1779-1798. 10.1084/jem.20160340.

169. Matta, S.K., Olias, P., Huang, Z., Wang, Q., Park, E., Yokoyama, W.M., and Sibley, L.D. (2019). *Toxoplasma gondii effector TgIST blocks type I interferon signaling to promote infection*. Proc Natl Acad Sci U S A ***116***, 17480-17491. 10.1073/pnas.1904637116.

170. Huang, Z., Liu, H., Nix, J., Xu, R., Knoverek, C.R., Bowman, G.R., Amarasinghe, G.K., and Sibley, L.D. (2022). *The intrinsically disordered protein TgIST from Toxoplasma gondii inhibits STAT1 signaling by blocking cofactor recruitment*. Nat Commun ***13***, 4047. 10.1038/s41467-022-31720-7.

171. Luder, C.G., Algner, M., Lang, C., Bleicher, N., and Gross, U. (2003). *Reduced expression of the inducible nitric oxide synthase after infection with Toxoplasma gondii facilitates parasite replication in activated murine macrophages*. Int J Parasitol ***33***, 833-844. 10.1016/s0020-7519(03)00092-4.

172. Bando, H., Sakaguchi, N., Lee, Y., Pradipta, A., Ma, J.S., Tanaka, S., Lai, D.H., Liu, J., Lun, Z.R., Nishikawa, Y., Sasai, M., and Yamamoto, M. (2018). *Toxoplasma Effector TgIST Targets Host IDO1 to Antagonize the IFN-gamma-Induced Anti-parasitic Response in Human Cells*. Front Immunol ***9***, 2073. 10.3389/fimmu.2018.02073.

173. Saeij, J.P., Boyle, J.P., Coller, S., Taylor, S., Sibley, L.D., Brooke-Powell, E.T., Ajioka, J.W., and Boothroyd, J.C. (2006). *Polymorphic secreted kinases are key virulence factors in toxoplasmosis*. Science ***314***, 1780-1783. 10.1126/science.1133690.

174. Alaganan, A., Fentress, S.J., Tang, K., Wang, Q., and Sibley, L.D. (2014). *Toxoplasma GRA7 effector increases turnover of immunity-related GTPases and contributes to acute virulence in the mouse*. Proc Natl Acad Sci U S A ***111***, 1126-1131. 10.1073/pnas.1313501111.

175. Behnke, M.S., Fentress, S.J., Mashayekhi, M., Li, L.X., Taylor, G.A., and Sibley, L.D. (2012). *The polymorphic pseudokinase ROP5 controls virulence in Toxoplasma gondii by regulating the active kinase ROP18*. PLoS Pathog ***8***, e1002992. 10.1371/journal.ppat.1002992.

176. Etheridge, R.D., Alaganan, A., Tang, K., Lou, H.J., Turk, B.E., and Sibley, L.D. (2014). *The Toxoplasma pseudokinase ROP5 forms complexes with ROP18 and ROP17 kinases that synergize to control acute virulence in mice*. Cell Host Microbe ***15***, 537-550. 10.1016/j.chom.2014.04.002.

177. Ihara, F., and Nishikawa, Y. (2021). *Toxoplasma gondii manipulates host cell signaling pathways via its secreted effector molecules*. Parasitol Int ***83***, 102368. 10.1016/j.parint.2021.102368.

178. Jensen, K.D., Hu, K., Whitmarsh, R.J., Hassan, M.A., Julien, L., Lu, D., Chen, L., Hunter, C.A., and Saeij, J.P. (2013). *Toxoplasma gondii rhoptry 16 kinase promotes host resistance to oral infection and intestinal inflammation only in the context of the dense granule protein GRA15*. Infect Immun ***81***, 2156-2167. 10.1128/IAI.01185-12.

179. Reese, M.L., Shah, N., and Boothroyd, J.C. (2014). *The Toxoplasma pseudokinase ROP5 is an allosteric inhibitor of the immunity-related GTPases*. J Biol Chem ***289***, 27849-27858. 10.1074/jbc.M114.567057.

180. Taylor, S., Barragan, A., Su, C., Fux, B., Fentress, S.J., Tang, K., Beatty, W.L., Hajj, H.E., Jerome, M., Behnke, M.S., White, M., Wootton, J.C., and Sibley, L.D. (2006). *A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen Toxoplasma gondii*. Science ***314***, 1776-1780. 10.1126/science.1133643.

181. Ten Hoeve, A.L., Braun, L., Rodriguez, M.E., Olivera, G.C., Bougdour, A., Belmudes, L., Coute, Y., Saeij, J.P.J., Hakimi, M.A., and Barragan, A. (2022). *The Toxoplasma effector GRA28 promotes parasite dissemination by inducing dendritic cell-like migratory properties in infected macrophages*. Cell Host Microbe ***30***, 1570-1588 e1577. 10.1016/j.chom.2022.10.001.

182. Shao, S., Sun, X., Chen, Y., Zhan, B., and Zhu, X. (2019). *Complement Evasion: An Effective Strategy That Parasites Utilize to Survive in the Host*. Front Microbiol ***10***, 532. 10.3389/fmicb.2019.00532.

183. Sikorski, P.M., Commodaro, A.G., and Grigg, M.E. (2021). *A Protective and Pathogenic Role for Complement During Acute Toxoplasma gondii Infection*. Front Cell Infect Microbiol ***11***, 634610. 10.3389/fcimb.2021.634610.

184. Mammari, N., Halabi, M.A., Yaacoub, S., Chlala, H., Darde, M.L., and Courtioux, B. (2019). *Toxoplasma gondii Modulates the Host Cell Responses: An Overview of Apoptosis Pathways*. Biomed Res Int ***2019***, 6152489. 10.1155/2019/6152489.

185. Lima, T.S., and Lodoen, M.B. (2019). *Mechanisms of Human Innate Immune Evasion by Toxoplasma gondii*. Front Cell Infect Microbiol ***9***, 103. 10.3389/fcimb.2019.00103.

186. Dostert, C., Grusdat, M., Letellier, E., and Brenner, D. (2019). *The TNF Family of Ligands and Receptors: Communication Modules in the Immune System and Beyond*. Physiol Rev ***99***, 115-160. 10.1152/physrev.00045.2017.

187. Hehlgans, T., and Pfeffer, K. (2005). *The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games*. Immunology ***115***, 1-20. 10.1111/j.1365-2567.2005.02143.x.

188. Albarbar, B., Dunnill, C., and Georgopoulos, N.T. (2015). *Regulation of cell fate by lymphotoxin (LT) receptor signalling: Functional differences and similarities of the LT system to other TNF superfamily (TNFSF) members*. Cytokine Growth Factor Rev ***26***, 659-671. 10.1016/j.cytogfr.2015.05.001.

189. Locksley, R.M., Killeen, N., and Lenardo, M.J. (2001). *The TNF and TNF receptor superfamilies: integrating mammalian biology*. Cell ***104***, 487-501. 10.1016/s0092-8674(01)00237-9.

190. Wang, Y.G., Kim, K.D., Wang, J., Yu, P., and Fu, Y.X. (2005). *Stimulating lymphotoxin beta receptor on the dendritic cells is critical for their homeostasis and expansion*. J Immunol ***175***, 6997-7002. 10.4049/jimmunol.175.10.6997.

191. Wimmer, N., Huber, B., Barabas, N., Rohrl, J., Pfeffer, K., and Hehlgans, T. (2012). *Lymphotoxin beta receptor activation on macrophages induces cross-tolerance to TLR4 and TLR9 ligands*. J Immunol ***188***, 3426-3433. 10.4049/jimmunol.1103324.

192. Wimmer, N., Huber, B., Wege, A.K., Barabas, N., Rohrl, J., Pfeffer, K., and Hehlgans, T. (2012). *Lymphotoxin-beta receptor activation on macrophages ameliorates acute DSS-induced intestinal inflammation in a TRIM30alpha-dependent manner*. Mol Immunol ***51***, 128-135. 10.1016/j.molimm.2012.02.118.

193. Riffelmacher, T., Giles, D.A., Zahner, S., Dicker, M., Andreyev, A.Y., McArdle, S., Perez-Jeldres, T., van der Gracht, E., Murray, M.P., Hartmann, N., Tumanov, A.V., and Kronenberg, M. (2021). *Metabolic activation and colitis pathogenesis is prevented by lymphotoxin beta receptor expression in neutrophils*. Mucosal Immunol ***14***, 679-690. 10.1038/s41385-021-00378-7.

194. Ware, C.F. (2005). *Network communications: lymphotoxins, LIGHT, and TNF*. Annu Rev Immunol ***23***, 787-819. 10.1146/annurev.immunol.23.021704.115719.

195. Force, W.R., Walter, B.N., Hession, C., Tizard, R., Kozak, C.A., Browning, J.L., and Ware, C.F. (1995). *Mouse lymphotoxin-beta receptor. Molecular genetics, ligand binding, and expression*. J Immunol ***155***, 5280-5288.

196. Kabashima, K., Banks, T.A., Ansel, K.M., Lu, T.T., Ware, C.F., and Cyster, J.G. (2005). *Intrinsic lymphotoxin-beta receptor requirement for homeostasis of lymphoid tissue dendritic cells*. Immunity ***22***, 439-450. 10.1016/j.immuni.2005.02.007.

197. De Trez, C. (2012). *Lymphotoxin-beta receptor expression and its related signaling pathways govern dendritic cell homeostasis and function*. Immunobiology ***217***, 1250-1258. 10.1016/j.imbio.2012.06.010.

198. Crowe, P.D., VanArsdale, T.L., Walter, B.N., Ware, C.F., Hession, C., Ehrenfels, B., Browning, J.L., Din, W.S., Goodwin, R.G., and Smith, C.A. (1994). *A lymphotoxin-beta-specific receptor*. Science ***264***, 707-710.

199. Zhai, Y., Guo, R., Hsu, T.L., Yu, G.L., Ni, J., Kwon, B.S., Jiang, G.W., Lu, J., Tan, J., Ugustus, M., Carter, K., Rojas, L., Zhu, F., Lincoln, C., Endress, G., Xing, L., Wang, S., Oh, K.O., Gentz, R., Ruben, S., Lippman, M.E., Hsieh, S.L., and Yang, D. (1998). *LIGHT, a novel ligand for lymphotoxin beta receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer*. J Clin Invest ***102***, 1142-1151. 10.1172/JCI3492.

200. Ware, C.F., Croft, M., and Neil, G.A. (2022). *Realigning the LIGHT signaling network to control dysregulated inflammation*. J Exp Med ***219***. 10.1084/jem.20220236.

201. Sudhamsu, J., Yin, J., Chiang, E.Y., Starovasnik, M.A., Grogan, J.L., and Hymowitz, S.G. (2013). *Dimerization of LTbetaR by LTalpha1beta2 is necessary and sufficient for signal transduction*. Proc Natl Acad Sci U S A ***110***, 19896-19901. 10.1073/pnas.1310838110.

202. Zotti, T., Scudiero, I., Vito, P., and Stilo, R. (2017). *The Emerging Role of TRAF7 in Tumor Development*. J Cell Physiol ***232***, 1233-1238. 10.1002/jcp.25676.

203. Park, H.H. (2018). *Structure of TRAF Family: Current Understanding of Receptor Recognition*. Front Immunol ***9***, 1999. 10.3389/fimmu.2018.01999.

204. Nakano, H., Oshima, H., Chung, W., Williams-Abbott, L., Ware, C.F., Yagita, H., and Okumura, K. (1996). *TRAF5, an activator of NF-kappaB and putative signal transducer for the lymphotoxin-beta receptor*. J Biol Chem ***271***, 14661-14664. 10.1074/jbc.271.25.14661.

205. Au, P.Y., and Yeh, W.C. (2007). *Physiological roles and mechanisms of signaling by TRAF2 and TRAF5*. Adv Exp Med Biol ***597***, 32-47. 10.1007/978-0-387-70630-6_3.

206. Bista, P., Zeng, W., Ryan, S., Bailly, V., Browning, J.L., and Lukashev, M.E. (2010). *TRAF3 controls activation of the canonical and alternative NFkappaB by the lymphotoxin beta receptor*. J Biol Chem ***285***, 12971-12978. 10.1074/jbc.M109.076091.

207. VanArsdale, T.L., VanArsdale, S.L., Force, W.R., Walter, B.N., Mosialos, G., Kieff, E., Reed, J.C., and Ware, C.F. (1997). *Lymphotoxin-beta receptor signaling complex: role of tumor necrosis factor receptor-associated factor 3 recruitment in cell death and activation of nuclear factor kappaB*. Proc Natl Acad Sci U S A ***94***, 2460-2465. 10.1073/pnas.94.6.2460.

208. Force, W.R., Cheung, T.C., and Ware, C.F. (1997). *Dominant negative mutants of TRAF3 reveal an important role for the coiled coil domains in cell death signaling by the lymphotoxin-beta receptor*. J Biol Chem ***272***, 30835-30840. 10.1074/jbc.272.49.30835.

209. Dadgostar, H., and Cheng, G. (2000). *Membrane localization of TRAF 3 enables JNK activation*. J Biol Chem ***275***, 2539-2544. 10.1074/jbc.275.4.2539.

210. Force, W.R., Glass, A.A., Benedict, C.A., Cheung, T.C., Lama, J., and Ware, C.F. (2000). *Discrete signaling regions in the lymphotoxin-beta receptor for tumor necrosis factor receptor-associated factor binding, subcellular localization, and activation of cell death and NF-kappaB pathways*. J Biol Chem ***275***, 11121-11129. 10.1074/jbc.275.15.11121.

211. Kim, Y.S., Nedospasov, S.A., and Liu, Z.G. (2005). *TRAF2 plays a key, nonredundant role in LIGHT-lymphotoxin beta receptor signaling*. Mol Cell Biol ***25***, 2130-2137. 10.1128/MCB.25.6.2130-2137.2005.

212. Remouchamps, C., Boutaffala, L., Ganeff, C., and Dejardin, E. (2011). *Biology and signal transduction pathways of the Lymphotoxin-alphabeta/LTbetaR system*. Cytokine Growth Factor Rev ***22***, 301-310. 10.1016/j.cytogfr.2011.11.007.

213. Papa, S., Bubici, C., Zazzeroni, F., Pham, C.G., Kuntzen, C., Knabb, J.R., Dean, K., and Franzoso, G. (2006). *The NF-kappaB-mediated control of the JNK cascade in the antagonism of programmed cell death in health and disease*. Cell Death Differ ***13***, 712-729. 10.1038/sj.cdd.4401865.

214. Tang, G., Minemoto, Y., Dibling, B., Purcell, N.H., Li, Z., Karin, M., and Lin, A. (2001). *Inhibition of JNK activation through NF-kappaB target genes*. Nature ***414***, 313-317. 10.1038/35104568.

215. He, J.Q., Saha, S.K., Kang, J.R., Zarnegar, B., and Cheng, G. (2007). *Specificity of TRAF3 in its negative regulation of the noncanonical NF-kappa B pathway*. J Biol Chem ***282***, 3688-3694. 10.1074/jbc.M610271200.

216. Hauer, J., Puschner, S., Ramakrishnan, P., Simon, U., Bongers, M., Federle, C., and Engelmann, H. (2005). *TNF receptor (TNFR)-associated factor (TRAF) 3 serves as an inhibitor of TRAF2/5-mediated activation of the noncanonical NF-kappaB pathway by TRAF-binding TNFRs*. Proc Natl Acad Sci U S A ***102***, 2874-2879. 10.1073/pnas.0500187102.

217. Sun, S.C. (2011). *Non-canonical NF-kappaB signaling pathway*. Cell Res ***21***, 71-85. 10.1038/cr.2010.177.

218. Pobezinskaya, Y.L., and Liu, Z. (2012). *The role of TRADD in death receptor signaling*. Cell Cycle ***11***, 871-876. 10.4161/cc.11.5.19300.

219. Cabal-Hierro, L., Rodriguez, M., Artime, N., Iglesias, J., Ugarte, L., Prado, M.A., and Lazo, P.S. (2014). *TRAF-mediated modulation of NF-kB AND JNK activation by TNFR2*. Cell Signal ***26***, 2658-2666. 10.1016/j.cellsig.2014.08.011.

220. Grell, M., Douni, E., Wajant, H., Lohden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., and Scheurich, P. (1995). *The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor*. Cell ***83***, 793-802. 10.1016/0092-8674(95)90192-2.

221. Rauert, H., Wicovsky, A., Muller, N., Siegmund, D., Spindler, V., Waschke, J., Kneitz, C., and Wajant, H. (2010). *Membrane tumor necrosis factor (TNF) induces p100 processing via TNF receptor-2 (TNFR2)*. J Biol Chem ***285***, 7394-7404. 10.1074/jbc.M109.037341.

222. Androlewicz, M.J., Browning, J.L., and Ware, C.F. (1992). *Lymphotoxin is expressed as a heteromeric complex with a distinct 33-kDa glycoprotein on the surface of an activated human T cell hybridoma*. J Biol Chem ***267***, 2542-2547.

223. Hayden, M.S., and Ghosh, S. (2012). *NF-kappaB, the first quarter-century: remarkable progress and outstanding questions*. Genes Dev ***26***, 203-234. 10.1101/gad.183434.111.

224. Mitchell, S., Vargas, J., and Hoffmann, A. (2016). *Signaling via the NFkappaB system*. Wiley Interdiscip Rev Syst Biol Med ***8***, 227-241. 10.1002/wsbm.1331.

225. Liu, T., Zhang, L., Joo, D., and Sun, S.C. (2017). *NF-kappaB signaling in inflammation*. Signal Transduct Target Ther ***2***, 17023-. 10.1038/sigtrans.2017.23.

226. Dejardin, E., Droin, N.M., Delhase, M., Haas, E., Cao, Y., Makris, C., Li, Z.W., Karin, M., Ware, C.F., and Green, D.R. (2002). *The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways*. Immunity ***17***, 525-535. 10.1016/s1074-7613(02)00423-5.

227. Muller, J.R., and Siebenlist, U. (2003). *Lymphotoxin beta receptor induces sequential activation of distinct NF-kappa B factors via separate signaling pathways*. J Biol Chem ***278***, 12006-12012. 10.1074/jbc.M210768200.

228. Solt, L.A., and May, M.J. (2008). *The IkappaB kinase complex: master regulator of NF-kappaB signaling*. Immunol Res ***42***, 3-18. 10.1007/s12026-008-8025-1.

229. Israel, A. (2010). *The IKK complex, a central regulator of NF-kappaB activation*. Cold Spring Harb Perspect Biol ***2***, a000158. 10.1101/cshperspect.a000158.

230. Liao, G., Zhang, M., Harhaj, E.W., and Sun, S.C. (2004). *Regulation of the NF-kappaB-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation*. J Biol Chem ***279***, 26243-26250. 10.1074/jbc.M403286200.

231. Xiao, G., Harhaj, E.W., and Sun, S.C. (2001). *NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100*. Mol Cell ***7***, 401-409. 10.1016/s1097-2765(01)00187-3.

232. Xiao, G., Fong, A., and Sun, S.C. (2004). *Induction of p100 processing by NF-kappaB-inducing kinase involves docking IkappaB kinase alpha (IKKalpha) to p100 and IKKalpha-mediated phosphorylation*. J Biol Chem ***279***, 30099-30105. 10.1074/jbc.M401428200.

233. Futterer, A., Mink, K., Luz, A., Kosco-Vilbois, M.H., and Pfeffer, K. (1998). *The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues*. Immunity ***9***, 59-70.

234. Shou, Y., Koroleva, E., Spencer, C.M., Shein, S.A., Korchagina, A.A., Yusoof, K.A., Parthasarathy, R., Leadbetter, E.A., Akopian, A.N., Munoz, A.R., and Tumanov, A.V. (2021). *Redefining the Role of Lymphotoxin Beta Receptor in the Maintenance of Lymphoid Organs and Immune Cell Homeostasis in Adulthood*. Front Immunol ***12***, 712632. 10.3389/fimmu.2021.712632.

235. Glatman Zaretsky, A., Silver, J.S., Siwicki, M., Durham, A., Ware, C.F., and Hunter, C.A. (2012). *Infection with Toxoplasma gondii alters lymphotoxin expression associated with changes in splenic architecture*. Infect Immun ***80***, 3602-3610. 10.1128/IAI.00333-12.

236. Mackay, F., Majeau, G.R., Lawton, P., Hochman, P.S., and Browning, J.L. (1997). *Lymphotoxin but not tumor necrosis factor functions to maintain splenic architecture and humoral responsiveness in adult mice*. Eur J Immunol ***27***, 2033-2042. 10.1002/eji.1830270830.

237. Fu, Y.X., Huang, G., Wang, Y., and Chaplin, D.D. (1998). *B lymphocytes induce the formation of follicular dendritic cell clusters in a lymphotoxin alpha-dependent fashion*. J Exp Med ***187***, 1009-1018. 10.1084/jem.187.7.1009.

238. Zindl, C.L., Kim, T.H., Zeng, M., Archambault, A.S., Grayson, M.H., Choi, K., Schreiber, R.D., and Chaplin, D.D. (2009). *The lymphotoxin LTalpha(1)beta(2) controls postnatal and adult spleen marginal sinus vascular structure and function*. Immunity ***30***, 408-420. 10.1016/j.immuni.2009.01.010.

239. Camara, A., Lavanant, A.C., Abe, J., Desforges, H.L., Alexandre, Y.O., Girardi, E., Igamberdieva, Z., Asano, K., Tanaka, M., Hehlgans, T., Pfeffer, K., Pfeffer, S., Mueller, S.N., Stein, J.V., and Mueller, C.G. (2022). *CD169(+) macrophages in lymph node and spleen critically depend on dual RANK and LTbetaR signaling*. Proc Natl Acad Sci U S A ***119***. 10.1073/pnas.2108540119.

240. De Togni, P., Goellner, J., Ruddle, N.H., Streeter, P.R., Fick, A., Mariathasan, S., Smith, S.C., Carlson, R., Shornick, L.P., Strauss-Schoenberger, J., and et al. (1994). *Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin*. Science ***264***, 703-707. 10.1126/science.8171322.

241. Alimzhanov, M.B., Kuprash, D.V., Kosco-Vilbois, M.H., Luz, A., Turetskaya, R.L., Tarakhovsky, A., Rajewsky, K., Nedospasov, S.A., and Pfeffer, K. (1997). *Abnormal development of secondary lymphoid tissues in lymphotoxin beta-deficient mice*. Proc Natl Acad Sci U S A ***94***, 9302-9307. 10.1073/pnas.94.17.9302.

242. Behnke, K., Sorg, U.R., Gabbert, H.E., and Pfeffer, K. (2017). *The Lymphotoxin beta Receptor Is Essential for Upregulation of IFN-Induced Guanylate-Binding Proteins and Survival after Toxoplasma gondii Infection*. Mediators Inflamm ***2017***, 7375818. 10.1155/2017/7375818.

243. Ngo, V.N., Korner, H., Gunn, M.D., Schmidt, K.N., Riminton, D.S., Cooper, M.D., Browning, J.L., Sedgwick, J.D., and Cyster, J.G. (1999). *Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen*. J Exp Med ***189***, 403-412. 10.1084/jem.189.2.403.

244. Wu, Q., Wang, Y., Wang, J., Hedgeman, E.O., Browning, J.L., and Fu, Y.X. (1999). *The requirement of membrane lymphotoxin for the presence of dendritic cells in lymphoid tissues*. J Exp Med ***190***, 629-638. 10.1084/jem.190.5.629.

245. Elewaut, D., Brossay, L., Santee, S.M., Naidenko, O.V., Burdin, N., De Winter, H., Matsuda, J., Ware, C.F., Cheroutre, H., and Kronenberg, M. (2000). *Membrane lymphotoxin is required for the development of different subpopulations of NK T cells*. J Immunol ***165***, 671-679. 10.4049/jimmunol.165.2.671.

246. Franki, A.S., Van Beneden, K., Dewint, P., Hammond, K.J., Lambrecht, S., Leclercq, G., Kronenberg, M., Deforce, D., and Elewaut, D. (2006). *A unique lymphotoxin alphabeta-dependent pathway regulates thymic emigration of Valpha14 invariant natural killer T cells*. Proc Natl Acad Sci U S A ***103***, 9160-9165. 10.1073/pnas.0508892103.

247. Iizuka, K., Chaplin, D.D., Wang, Y., Wu, Q., Pegg, L.E., Yokoyama, W.M., and Fu, Y.X. (1999). *Requirement for membrane lymphotoxin in natural killer cell development*. Proc Natl Acad Sci U S A ***96***, 6336-6340. 10.1073/pnas.96.11.6336.

248. Tersteegen, A., Sorg, U.R., Virgen-Slane, R., Helle, M., Petzsch, P., Dunay, I.R., Kohrer, K., Degrandi, D., Ware, C.F., and Pfeffer, K. (2021). *Lymphotoxin beta Receptor: a Crucial Role in Innate and Adaptive Immune Responses against Toxoplasma gondii*. Infect Immun ***89***. 10.1128/IAI.00026-21.

249. Borelli, A., and Irla, M. (2021). *Lymphotoxin: from the physiology to the regeneration of the thymic function*. Cell Death Differ ***28***, 2305-2314. 10.1038/s41418-021-00834-8.

250. Nitta, T., Tsutsumi, M., Nitta, S., Muro, R., Suzuki, E.C., Nakano, K., Tomofuji, Y., Sawa, S., Okamura, T., Penninger, J.M., and Takayanagi, H. (2020). *Fibroblasts as a source of self-antigens for central immune tolerance*. Nat Immunol ***21***, 1172-1180. 10.1038/s41590-020-0756-8.

251. Chin, R.K., Lo, J.C., Kim, O., Blink, S.E., Christiansen, P.A., Peterson, P., Wang, Y., Ware, C., and Fu, Y.X. (2003). *Lymphotoxin pathway directs thymic Aire expression*. Nat Immunol ***4***, 1121-1127. 10.1038/ni982.

252. Boehm, T., Scheu, S., Pfeffer, K., and Bleul, C.C. (2003). *Thymic medullary epithelial cell differentiation, thymocyte emigration, and the control of autoimmunity require lympho-epithelial cross talk via LTbetaR*. J Exp Med ***198***, 757-769. 10.1084/jem.20030794.

253. Cosway, E.J., Lucas, B., James, K.D., Parnell, S.M., Carvalho-Gaspar, M., White, A.J., Tumanov, A.V., Jenkinson, W.E., and Anderson, G. (2017). *Redefining thymus medulla specialization for central tolerance*. J Exp Med ***214***, 3183-3195. 10.1084/jem.20171000.

254. Seleznik, G., Seeger, H., Bauer, J., Fu, K., Czerkowicz, J., Papandile, A., Poreci, U., Rabah, D., Ranger, A., Cohen, C.D., Lindenmeyer, M., Chen, J., Edenhofer, I., Anders, H.J., Lech, M., Wuthrich, R.P., Ruddle, N.H., Moeller, M.J., Kozakowski, N., Regele, H., Browning, J.L., Heikenwalder, M., and Segerer, S. (2016). *The lymphotoxin beta receptor is a potential therapeutic target in renal inflammation*. Kidney Int ***89***, 113-126. 10.1038/ki.2015.280.

255. Zhang, G., Feizi, N., Zhao, D., Halesha, L., Williams, A.L., Randhawa, P.S., Abou-Daya, K.I., and Oberbarnscheidt, M.H. (2024). *Lymphotoxin beta receptor and tertiary lymphoid organs shape acute and chronic allograft rejection*. JCI Insight. 10.1172/jci.insight.177555.

256. Liu, H., Dai, H., Qiu, F., Chen, Y., Liang, C.L., Yang, B., Gong, N., Bromberg, J.S., and Dai, Z. (2024). *Electrostimulation suppresses allograft rejection via promoting lymphatic regulatory T cell migration mediated by lymphotoxin (LT)-LTbeta receptor signaling*. Am J Transplant. 10.1016/j.ajt.2024.06.019.

257. Fernandes, M.T., Dejardin, E., and dos Santos, N.R. (2016). *Context-dependent roles for lymphotoxin-beta receptor signaling in cancer development*. Biochim Biophys Acta ***1865***, 204-219. 10.1016/j.bbcan.2016.02.005.

258. Wolf, M.J., Seleznik, G.M., Zeller, N., and Heikenwalder, M. (2010). *The unexpected role of lymphotoxin beta receptor signaling in carcinogenesis: from lymphoid tissue formation to liver and prostate cancer development*. Oncogene ***29***, 5006-5018. 10.1038/onc.2010.260.

259. Ehlers, S., Holscher, C., Scheu, S., Tertilt, C., Hehlgans, T., Suwinski, J., Endres, R., and Pfeffer, K. (2003). *The lymphotoxin beta receptor is critically involved in controlling infections with the intracellular pathogens Mycobacterium tuberculosis and Listeria monocytogenes*. J Immunol ***170***, 5210-5218.

260. Kutsch, S., Degrandi, D., and Pfeffer, K. (2008). *Immediate lymphotoxin beta receptor-mediated transcriptional response in host defense against L. monocytogenes*. Immunobiology ***213***, 353-366. 10.1016/j.imbio.2007.10.011.

261. Kursar, M., Janner, N., Pfeffer, K., Brinkmann, V., Kaufmann, S.H., and Mittrucker, H.W. (2008). *Requirement of secondary lymphoid tissues for the induction of primary and secondary T cell responses against Listeria monocytogenes*. Eur J Immunol ***38***, 127-138. 10.1002/eji.200737142.

262. Spahn, T.W., Maaser, C., Eckmann, L., Heidemann, J., Lugering, A., Newberry, R., Domschke, W., Herbst, H., and Kucharzik, T. (2004). *The lymphotoxin-beta receptor is critical for control of murine Citrobacter rodentium-induced colitis*. Gastroenterology ***127***, 1463-1473. 10.1053/j.gastro.2004.08.022.

263. Wang, Y., Koroleva, E.P., Kruglov, A.A., Kuprash, D.V., Nedospasov, S.A., Fu, Y.X., and Tumanov, A.V. (2010). *Lymphotoxin beta receptor signaling in intestinal epithelial cells orchestrates innate immune responses against mucosal bacterial infection*. Immunity ***32***, 403-413. 10.1016/j.immuni.2010.02.011.

264. Banks, T.A., Rickert, S., and Ware, C.F. (2006). *Restoring immune defenses via lymphotoxin signaling: lessons from cytomegalovirus*. Immunol Res ***34***, 243-254. 10.1385/IR:34:3:243.

265. Jin, L., Guo, X., Shen, C., Hao, X., Sun, P., Li, P., Xu, T., Hu, C., Rose, O., Zhou, H., Yang, M., Qin, C.F., Guo, J., Peng, H., Zhu, M., Cheng, G., Qi, X., and Lai, R. (2018). *Salivary factor LTRIN from Aedes aegypti facilitates the transmission of Zika virus by interfering with the lymphotoxin-beta receptor*. Nat Immunol ***19***, 342-353. 10.1038/s41590-018-0063-9.

266. Xu, G., Liu, D., Okwor, I., Wang, Y., Korner, H., Kung, S.K., Fu, Y.X., and Uzonna, J.E. (2007). *LIGHT Is critical for IL-12 production by dendritic cells, optimal CD4+ Th1 cell response, and resistance to Leishmania major*. J Immunol ***179***, 6901-6909. 10.4049/jimmunol.179.10.6901.

267. Stanley, A.C., de Labastida Rivera, F., Haque, A., Sheel, M., Zhou, Y., Amante, F.H., Bunn, P.T., Randall, L.M., Pfeffer, K., Scheu, S., Hickey, M.J., Saunders, B.M., Ware, C., Hill, G.R., Tamada, K., Kaye, P.M., and Engwerda, C.R. (2011). *Critical roles for LIGHT and its receptors in generating T cell-mediated immunity during Leishmania donovani infection*. PLoS Pathog ***7***, e1002279. 10.1371/journal.ppat.1002279.

268. Krucken, J., Braun, J.V., Dkhil, M.A., Grunwald, A., and Wunderlich, F. (2005). *Deletion of LTbetaR augments male susceptibility to Plasmodium chabaudi*. Parasite Immunol ***27***, 205-212. 10.1111/j.1365-3024.2005.00763.x.

269. Randall, L.M., Amante, F.H., Zhou, Y., Stanley, A.C., Haque, A., Rivera, F., Pfeffer, K., Scheu, S., Hill, G.R., Tamada, K., and Engwerda, C.R. (2008). *Cutting edge: selective blockade of LIGHT-lymphotoxin beta receptor signaling protects mice from experimental cerebral malaria caused by Plasmodium berghei ANKA*. J Immunol ***181***, 7458-7462. 10.4049/jimmunol.181.11.7458.

270. Schneider, K., Loewendorf, A., De Trez, C., Fulton, J., Rhode, A., Shumway, H., Ha, S., Patterson, G., Pfeffer, K., Nedospasov, S.A., Ware, C.F., and Benedict, C.A. (2008). *Lymphotoxin-mediated crosstalk between B cells and splenic stroma promotes the initial type I interferon response to cytomegalovirus*. Cell Host Microbe ***3***, 67-76. 10.1016/j.chom.2007.12.008.

271. Vu, F., Dianzani, U., Ware, C.F., Mak, T., and Gommerman, J.L. (2008). *ICOS, CD40, and lymphotoxin beta receptors signal sequentially and interdependently to initiate a germinal center reaction*. J Immunol ***180***, 2284-2293. 10.4049/jimmunol.180.4.2284.

272. Myers, R.C., King, R.G., Carter, R.H., and Justement, L.B. (2013). *Lymphotoxin alpha1beta2 expression on B cells is required for follicular dendritic cell activation during the germinal center response*. Eur J Immunol ***43***, 348-359. 10.1002/eji.201242471.

273. Kang, H., Remington, J.S., and Suzuki, Y. (2000). *Decreased resistance of B cell-deficient mice to infection with Toxoplasma gondii despite unimpaired expression of IFN-gamma, TNF-alpha, and inducible nitric oxide synthase*. J Immunol ***164***, 2629-2634. 10.4049/jimmunol.164.5.2629.

274. Souza, S.P., Splitt, S.D., Sanchez-Arcila, J.C., Alvarez, J.A., Wilson, J.N., Wizzard, S., Luo, Z., Baumgarth, N., and Jensen, K.D.C. (2021). *Genetic mapping reveals Nfkbid as a central regulator of humoral immunity to Toxoplasma gondii*. PLoS Pathog ***17***, e1010081. 10.1371/journal.ppat.1010081.

275. Sayles, P.C., Gibson, G.W., and Johnson, L.L. (2000). *B cells are essential for vaccination-induced resistance to virulent Toxoplasma gondii*. Infect Immun ***68***, 1026-1033. 10.1128/IAI.68.3.1026-1033.2000.

276. Chen, M., Mun, H.S., Piao, L.X., Aosai, F., Norose, K., Mohamed, R.M., Belal, U.S., Fang, H., Ahmed, A.K., Kang, H.K., Matsuzaki, G., Kitamura, D., and Yano, A. (2003). *Induction of protective immunity by primed B-1 cells in Toxoplasma gondii -infected B cell-deficient mice*. Microbiol Immunol ***47***, 997-1003. 10.1111/j.1348-0421.2003.tb03460.x.

277. Cariappa, A., Mazo, I.B., Chase, C., Shi, H.N., Liu, H., Li, Q., Rose, H., Leung, H., Cherayil, B.J., Russell, P., von Andrian, U., and Pillai, S. (2005). *Perisinusoidal B cells in the bone marrow participate in T-independent responses to blood-borne microbes*. Immunity ***23***, 397-407. 10.1016/j.immuni.2005.09.004.

278. Sapoznikov, A., Pewzner-Jung, Y., Kalchenko, V., Krauthgamer, R., Shachar, I., and Jung, S. (2008). *Perivascular clusters of dendritic cells provide critical survival signals to B cells in bone marrow niches*. Nat Immunol ***9***, 388-395. 10.1038/ni1571.

279. Pillai, S., and Cariappa, A. (2009). *The bone marrow perisinusoidal niche for recirculating B cells and the positive selection of bone marrow-derived B lymphocytes*. Immunol Cell Biol ***87***, 16-19. 10.1038/icb.2008.89.

280. Gore, Y., Starlets, D., Maharshak, N., Becker-Herman, S., Kaneyuki, U., Leng, L., Bucala, R., and Shachar, I. (2008). *Macrophage migration inhibitory factor induces B cell survival by activation of a CD74-CD44 receptor complex*. J Biol Chem ***283***, 2784-2792. 10.1074/jbc.M703265200.

281. Klasen, C., Ziehm, T., Huber, M., Asare, Y., Kapurniotu, A., Shachar, I., Bernhagen, J., and El Bounkari, O. (2018). *LPS-mediated cell surface expression of CD74 promotes the proliferation of B cells in response to MIF*. Cell Signal ***46***, 32-42. 10.1016/j.cellsig.2018.02.010.

282. Pereira, J.P., An, J., Xu, Y., Huang, Y., and Cyster, J.G. (2009). *Cannabinoid receptor 2 mediates the retention of immature B cells in bone marrow sinusoids*. Nat Immunol ***10***, 403-411. 10.1038/ni.1710.

283. Ueda, Y., Yang, K., Foster, S.J., Kondo, M., and Kelsoe, G. (2004). *Inflammation controls B lymphopoiesis by regulating chemokine CXCL12 expression*. J Exp Med ***199***, 47-58. 10.1084/jem.20031104.

284. Griffiths, S.D., Goodhead, D.T., Marsden, S.J., Wright, E.G., Krajewski, S., Reed, J.C., Korsmeyer, S.J., and Greaves, M. (1994). *Interleukin 7-dependent B lymphocyte precursor cells are ultrasensitive to apoptosis*. J Exp Med ***179***, 1789-1797. 10.1084/jem.179.6.1789.

285. Stopfer, P., Obermeier, F., Dunger, N., Falk, W., Farkas, S., Janotta, M., Moller, A., Mannel, D.N., and Hehlgans, T. (2004). *Blocking lymphotoxin-beta receptor activation diminishes inflammation via reduced mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expression and leucocyte margination in chronic DSS-induced colitis*. Clin Exp Immunol ***136***, 21-29. 10.1111/j.1365-2249.2004.02402.x.

286. Browning, J.L., Allaire, N., Ngam-Ek, A., Notidis, E., Hunt, J., Perrin, S., and Fava, R.A. (2005). *Lymphotoxin-beta receptor signaling is required for the homeostatic control of HEV differentiation and function*. Immunity ***23***, 539-550. 10.1016/j.immuni.2005.10.002.

287. Madge, L.A., Kluger, M.S., Orange, J.S., and May, M.J. (2008). *Lymphotoxin-alpha 1 beta 2 and LIGHT induce classical and noncanonical NF-kappa B-dependent proinflammatory gene expression in vascular endothelial cells*. J Immunol ***180***, 3467-3477. 10.4049/jimmunol.180.5.3467.

288. Ware, C.F. (2008). *Targeting lymphocyte activation through the lymphotoxin and LIGHT pathways*. Immunol Rev ***223***, 186-201. 10.1111/j.1600-065X.2008.00629.x.

289. Bielecki, P., Gindzienska-Sieskiewicz, E., Reszec, J., Piszczatowski, B., Rogowski, M., Kowal-Bielecka, O., Kowal, K., and Sieskiewicz, A. (2021). *Expression of LIGHT/TNFSF14 and Its Receptors, HVEM and LTbetaR, Correlates with the Severity of Fibrosis in Lacrimal Sacs from Patients with Lacrimal Duct Obstruction*. Ophthalmol Ther ***10***, 63-74. 10.1007/s40123-020-00320-3.

290. Savage, A.K., Gutschow, M.V., Chiang, T., Henderson, K., Green, R., Chaudhari, M., Swanson, E., Heubeck, A.T., Kondza, N., Burley, K.C., Genge, P.C., Lord, C., Smith, T., Thomson, Z., Beaubien, A., Johnson, E., Goldy, J., Bolouri, H., Buckner, J.H., Meijer, P., Coffey, E.M., Skene, P.J., Torgerson, T.R., Li, X.J., and Bumol, T.F. (2021). *Multimodal analysis for human ex vivo studies shows extensive molecular changes from delays in blood processing*. iScience ***24***, 102404. 10.1016/j.isci.2021.102404.

291. van Hooren, L., Vaccaro, A., Ramachandran, M., Vazaios, K., Libard, S., van de Walle, T., Georganaki, M., Huang, H., Pietila, I., Lau, J., Ulvmar, M.H., Karlsson, M.C.I., Zetterling, M., Mangsbo, S.M., Jakola, A.S., Olsson Bontell, T., Smits, A., Essand, M., and Dimberg, A. (2021). *Agonistic CD40 therapy induces tertiary lymphoid structures but impairs responses to checkpoint blockade in glioma*. Nat Commun ***12***, 4127. 10.1038/s41467-021-24347-7.

292. Duhen, T., Pasero, C., Mallet, F., Barbarat, B., Olive, D., and Costello, R.T. (2004). *LIGHT costimulates CD40 triggering and induces immunoglobulin secretion; a novel key partner in T cell-dependent B cell terminal differentiation*. Eur J Immunol ***34***, 3534-3541. 10.1002/eji.200425598.

293. Ansel, K.M., Ngo, V.N., Hyman, P.L., Luther, S.A., Forster, R., Sedgwick, J.D., Browning, J.L., Lipp, M., and Cyster, J.G. (2000). *A chemokine-driven positive feedback loop organizes lymphoid follicles*. Nature ***406***, 309-314. 10.1038/35018581.

294. Ngo, V.N., Cornall, R.J., and Cyster, J.G. (2001). *Splenic T zone development is B cell dependent*. J Exp Med ***194***, 1649-1660. 10.1084/jem.194.11.1649.

295. Tumanov, A., Kuprash, D., Lagarkova, M., Grivennikov, S., Abe, K., Shakhov, A., Drutskaya, L., Stewart, C., Chervonsky, A., and Nedospasov, S. (2002). *Distinct role of surface lymphotoxin expressed by B cells in the organization of secondary lymphoid tissues*. Immunity ***17***, 239-250. 10.1016/s1074-7613(02)00397-7.

296. Wijdeven, R.H., van Luijn, M.M., Wierenga-Wolf, A.F., Akkermans, J.J., van den Elsen, P.J., Hintzen, R.Q., and Neefjes, J. (2018). *Chemical and genetic control of IFNgamma-induced MHCII expression*. EMBO Rep ***19***. 10.15252/embr.201745553.

297. Fonseca, M.I., Carpenter, P.M., Park, M., Palmarini, G., Nelson, E.L., and Tenner, A.J. (2001). *C1qR(P), a myeloid cell receptor in blood, is predominantly expressed on endothelial cells in human tissue*. J Leukoc Biol ***70***, 793-800.

298. Norsworthy, P.J., Fossati-Jimack, L., Cortes-Hernandez, J., Taylor, P.R., Bygrave, A.E., Thompson, R.D., Nourshargh, S., Walport, M.J., and Botto, M. (2004). *Murine CD93 (C1qRp) contributes to the removal of apoptotic cells in vivo but is not required for C1q-mediated enhancement of phagocytosis*. J Immunol ***172***, 3406-3414. 10.4049/jimmunol.172.6.3406.

299. Wu, Z., Zheng, Y., Sheng, J., Han, Y., Yang, Y., Pan, H., and Yao, J. (2022). *CD3(+)CD4(-)CD8(-) (Double-Negative) T Cells in Inflammation, Immune Disorders and Cancer*. Front Immunol ***13***, 816005. 10.3389/fimmu.2022.816005.

300. Hu, S.H., Zhang, L.H., Gao, J., Guo, J.H., Xun, X.D., Xiang, X., Cheng, Q., Li, Z., and Zhu, J.Y. (2021). *NKG2D Enhances Double-Negative T Cell Regulation of B Cells*. Front Immunol ***12***, 650788. 10.3389/fimmu.2021.650788.

301. Brinkman, C.C., Iwami, D., Hritzo, M.K., Xiong, Y., Ahmad, S., Simon, T., Hippen, K.L., Blazar, B.R., and Bromberg, J.S. (2016). *Treg engage lymphotoxin beta receptor for afferent lymphatic transendothelial migration*. Nat Commun ***7***, 12021. 10.1038/ncomms12021.

302. Saxena, V., Piao, W., Li, L., Paluskievicz, C., Xiong, Y., Simon, T., Lakhan, R., Brinkman, C.C., Walden, S., Hippen, K.L., WillsonShirkey, M., Lee, Y.S., Wagner, C., Blazar, B.R., and Bromberg, J.S. (2022). *Treg tissue stability depends on lymphotoxin beta-receptor- and adenosine-receptor-driven lymphatic endothelial cell responses*. Cell Rep ***39***, 110727. 10.1016/j.celrep.2022.110727.

303. El Bissati, K., Krishack, P.A., Zhou, Y., Weber, C.R., Lykins, J., Jankovic, D., Edelblum, K.L., Fraczek, L., Grover, H., Chentoufi, A.A., Singh, G., Reardon, C., Dubey, J.P., Reed, S., Alexander, J., Sidney, J., Sette, A., Shastri, N., and McLeod, R. (2023). *CD4(+) T Cell Responses to Toxoplasma gondii Are a Double-Edged Sword*. Vaccines (Basel) ***11***. 10.3390/vaccines11091485.

304. Lopez-Yglesias, A.H., Burger, E., Araujo, A., Martin, A.T., and Yarovinsky, F. (2018). *T-bet-independent Th1 response induces intestinal immunopathology during Toxoplasma gondii infection*. Mucosal Immunol ***11***, 921-931. 10.1038/mi.2017.102.

305. Olguin, J.E., Fernandez, J., Salinas, N., Juarez, I., Rodriguez-Sosa, M., Campuzano, J., Castellanos, C., and Saavedra, R. (2015). *Adoptive transfer of CD4(+)Foxp3(+) regulatory T cells to C57BL/6J mice during acute infection with Toxoplasma gondii down modulates the exacerbated Th1 immune response*. Microbes Infect ***17***, 586-595. 10.1016/j.micinf.2015.04.002.

306. Elias, S., Sharma, R., Schizas, M., Valdez, I., Rampersaud, S., Park, S.M., Gonzalez-Figueroa, P., Li, Q.Z., Hoyos, B., and Rudensky, A.Y. (2022). *CXCR4+ Treg cells control serum IgM levels and natural IgM autoantibody production by B-1 cells in the bone marrow*. J Exp Med ***219***. 10.1084/jem.20220047.

307. Pavia, C.S., Bittker, S.J., and Curnick, K.E. (1992). *Passive immunization protects guinea pigs from lethal Toxoplasma infection*. FEMS Microbiol Immunol ***4***, 97-104. 10.1111/j.1574-6968.1992.tb04975.x.

308. Mineo, J.R., McLeod, R., Mack, D., Smith, J., Khan, I.A., Ely, K.H., and Kasper, L.H. (1993). *Antibodies to Toxoplasma gondii major surface protein (SAG-1, P30) inhibit infection of host cells and are produced in murine intestine after peroral infection*. J Immunol ***150***, 3951-3964.

309. Cha, D.Y., Song, I.K., Lee, G.S., Hwang, O.S., Noh, H.J., Yeo, S.D., Shin, D.W., and Lee, Y.H. (2001). *Effects of specific monoclonal antibodies to dense granular proteins on the invasion of Toxoplasma gondii in vitro and in vivo*. Korean J Parasitol ***39***, 233-240. 10.3347/kjp.2001.39.3.233.

310. Johnson, L.L., and Sayles, P.C. (2002). *Deficient humoral responses underlie susceptibility to Toxoplasma gondii in CD4-deficient mice*. Infect Immun ***70***, 185-191. 10.1128/IAI.70.1.185-191.2002.

311. Johnson, L.L., Lanthier, P., Hoffman, J., and Chen, W. (2004). *Vaccination protects B cell-deficient mice against an oral challenge with mildly virulent Toxoplasma gondii*. Vaccine ***22***, 4054-4061. 10.1016/j.vaccine.2004.03.056.

312. Kang, H.J., Kim, M.J., Chu, K.B., Lee, S.H., Moon, E.K., and Quan, F.S. (2021). *Passive Immunity and Antibody Response Induced by Toxoplasma gondii VLP Immunization*. Vaccines (Basel) ***9***. 10.3390/vaccines9050425.

313. Brinkmann, V., Remington, J.S., and Sharma, S.D. (1993). *Vaccination of mice with the protective F3G3 antigen of Toxoplasma gondii activates CD4+ but not CD8+ T cells and induces Toxoplasma specific IgG antibody*. Mol Immunol ***30***, 353-358. 10.1016/0161-5890(93)90064-i.

314. Sharma, S.D., Araujo, F.G., and Remington, J.S. (1984). *Toxoplasma antigen isolated by affinity chromatography with monoclonal antibody protects mice against lethal infection with Toxoplasma gondii*. J Immunol ***133***, 2818-2820.

315. Wu, M., Liu, S., Chen, Y., Liu, D., An, R., Cai, H., Wang, J., Zhou, N., Obed, C., Han, M., Shen, J., Chen, L., and Du, J. (2022). *Live-attenuated ME49Deltacdpk3 strain of Toxoplasma gondii protects against acute and chronic toxoplasmosis*. NPJ Vaccines ***7***, 98. 10.1038/s41541-022-00518-5.

316. Zhang, H.S., Cao, H., Li, C.X., Zhang, Z.W., Wang, M., Zhu, X.Q., and Zheng, X.N. (2024). *Immunization with Live-Attenuated RHDeltahad2a Strain Confers Partial Protective Immunity against Acute and Chronic Infection of Toxoplasma gondii in Mice*. Pathogens ***13***. 10.3390/pathogens13020121.

317. Yang, W.B., Wang, J.L., Gui, Q., Zou, Y., Chen, K., Liu, Q., Liang, Q.L., Zhu, X.Q., and Zhou, D.H. (2019). *Immunization With a Live-Attenuated RH:DeltaNPT1 Strain of Toxoplasma gondii Induces Strong Protective Immunity Against Toxoplasmosis in Mice*. Front Microbiol ***10***, 1875. 10.3389/fmicb.2019.01875.

318. Couper, K.N., Roberts, C.W., Brombacher, F., Alexander, J., and Johnson, L.L. (2005). *Toxoplasma gondii-specific immunoglobulin M limits parasite dissemination by preventing host cell invasion*. Infect Immun ***73***, 8060-8068. 10.1128/IAI.73.12.8060-8068.2005.

319. Anderson, S.E., Jr., Bautista, S.C., and Remington, J.S. (1976). *Specific antibody-dependent killing of Toxoplasma gondii by normal macrophages*. Clin Exp Immunol ***26***, 375-380.

320. Erbe, D.V., Pfefferkorn, E.R., and Fanger, M.W. (1991). *Functions of the various IgG Fc receptors in mediating killing of Toxoplasma gondii*. J Immunol ***146***, 3145-3151.

321. Hammouda, N.A., Abo el-Naga, I., Hussein, E.D., and Rashwan, E.A. (1995). *Opsonization and intracellular killing of Toxoplasma gondii by human mononuclear phagocytes*. J Egypt Soc Parasitol ***25***, 11-17.

322. Joiner, K.A., Fuhrman, S.A., Miettinen, H.M., Kasper, L.H., and Mellman, I. (1990). *Toxoplasma gondii: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts*. Science ***249***, 641-646. 10.1126/science.2200126.

323. Vercammen, M., Scorza, T., El Bouhdidi, A., Van Beeck, K., Carlier, Y., Dubremetz, J.F., and Verschueren, H. (1999). *Opsonization of Toxoplasma gondii tachyzoites with nonspecific immunoglobulins promotes their phagocytosis by macrophages and inhibits their proliferation in nonphagocytic cells in tissue culture*. Parasite Immunol ***21***, 555-563. 10.1046/j.1365-3024.1999.00256.x.

324. Foster, B.G., and McCulloch, W.F. (1968). *Studies of active and passive immunity in animals inoculated with Toxoplasma gondii*. Can J Microbiol ***14***, 103-110. 10.1139/m68-018.

325. Strickland, G.T., and Sayles, P.C. (1977). *Depressed antibody responses to a thymus-dependent antigen in toxoplasmosis*. Infect Immun ***15***, 184-190. 10.1128/iai.15.1.184-190.1977.

326. Masihi, K.N., and Werner, H. (1977). *Suppression and enhancement of humoral immune response to Toxoplasma gondii by passive antibody*. Experientia ***33***, 1586-1587. 10.1007/BF01934012.

327. Chu, K.B., and Quan, F.S. (2021). *Advances in Toxoplasma gondii Vaccines: Current Strategies and Challenges for Vaccine Development*. Vaccines (Basel) ***9***. 10.3390/vaccines9050413.

328. Zahavi, D., AlDeghaither, D., O'Connell, A., and Weiner, L.M. (2018). *Enhancing antibody-dependent cell-mediated cytotoxicity: a strategy for improving antibody-based immunotherapy*. Antib Ther ***1***, 7-12. 10.1093/abt/tby002.

329. Suzuki, Y., Joh, K., and Kobayashi, A. (1987). *Macrophage-mediated suppression of immune responses in Toxoplasma-infected mice. III. Suppression of antibody responses to parasite itself*. Cell Immunol ***110***, 218-225. 10.1016/0008-8749(87)90117-1.

330. Suzuki, Y., and Kobayashi, A. (1984). *Macrophage-mediated suppression of immune responses in Toxoplasma-infected mice. I. Inhibition of proliferation of lymphocytes in primary antibody responses*. Cell Immunol ***85***, 417-427. 10.1016/0008-8749(84)90255-7.

331. Suzuki, Y., Watanabe, N., and Kobayashi, A. (1981). *Nonspecific suppression of primary antibody responses and presence of plastic-adherent suppressor cells in Toxoplasma gondii-infected mice*. Infect Immun ***34***, 30-35. 10.1128/iai.34.1.30-35.1981.

332. Suzuki, Y., and Kobayashi, A. (1985). *Macrophage-mediated suppression of immune responses in Toxoplasma-infected mice. II. Both H-2-linked and -nonlinked control of induction of suppressor macrophages*. Cell Immunol ***91***, 375-384. 10.1016/0008-8749(85)90235-7.

333. Sultana, M.A., Du, A., Carow, B., Angbjar, C.M., Weidner, J.M., Kanatani, S., Fuks, J.M., Muliaditan, T., James, J., Mansfield, I.O., Campbell, T.M., Liu, L., Kadri, N., Lambert, H., Barragan, A., and Chambers, B.J. (2017). *Downmodulation of Effector Functions in NK Cells upon Toxoplasma gondii Infection*. Infect Immun ***85***. 10.1128/IAI.00069-17.

334. Zhu, W., Li, J., Pappoe, F., Shen, J., and Yu, L. (2019). *Strategies Developed by Toxoplasma gondii to Survive in the Host*. Front Microbiol ***10***, 899. 10.3389/fmicb.2019.00899.

335. Channon, J.Y., and Kasper, L.H. (1996). *Toxoplasma gondii-induced immune suppression by human peripheral blood monocytes: role of gamma interferon*. Infect Immun ***64***, 1181-1189. 10.1128/iai.64.4.1181-1189.1996.

336. Zuniga, E., Acosta-Rodriguez, E., Merino, M.C., Montes, C., and Gruppi, A. (2005). *Depletion of immature B cells during Trypanosoma cruzi infection: involvement of myeloid cells and the cyclooxygenase pathway*. Eur J Immunol ***35***, 1849-1858. 10.1002/eji.200526005.

337. Zuniga, E., Motran, C.C., Montes, C.L., Yagita, H., and Gruppi, A. (2002). *Trypanosoma cruzi infection selectively renders parasite-specific IgG+ B lymphocytes susceptible to Fas/Fas ligand-mediated fratricide*. J Immunol ***168***, 3965-3973. 10.4049/jimmunol.168.8.3965.

338. Bockstal, V., Geurts, N., and Magez, S. (2011). *Acute Disruption of Bone Marrow B Lymphopoiesis and Apoptosis of Transitional and Marginal Zone B Cells in the Spleen following a Blood-Stage Plasmodium chabaudi Infection in Mice*. J Parasitol Res ***2011***, 534697. 10.1155/2011/534697.

339. Bockstal, V., Guirnalda, P., Caljon, G., Goenka, R., Telfer, J.C., Frenkel, D., Radwanska, M., Magez, S., and Black, S.J. (2011). *T. brucei infection reduces B lymphopoiesis in bone marrow and truncates compensatory splenic lymphopoiesis through transitional B-cell apoptosis*. PLoS Pathog ***7***, e1002089. 10.1371/journal.ppat.1002089.

340. Amezcua Vesely, M.C., Bermejo, D.A., Montes, C.L., Acosta-Rodriguez, E.V., and Gruppi, A. (2012). *B-Cell Response during Protozoan Parasite Infections*. J Parasitol Res ***2012***, 362131. 10.1155/2012/362131.

341. Xu, H., and Heyman, B. (2020). *IgG-mediated suppression of antibody responses: Hiding or snatching epitopes?* Scand J Immunol ***92***, e12921. 10.1111/sji.12921.

342. Taborda, C.P., Rivera, J., Zaragoza, O., and Casadevall, A. (2003). *More is not necessarily better: prozone-like effects in passive immunization with IgG*. J Immunol ***170***, 3621-3630. 10.4049/jimmunol.170.7.3621.

343. Baumgarth, N., Herman, O.C., Jager, G.C., Brown, L.E., Herzenberg, L.A., and Chen, J. (2000). *B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection*. J Exp Med ***192***, 271-280. 10.1084/jem.192.2.271.

344. Choi, Y.S., Dieter, J.A., Rothaeusler, K., Luo, Z., and Baumgarth, N. (2012). *B-1 cells in the bone marrow are a significant source of natural IgM*. Eur J Immunol ***42***, 120-129. 10.1002/eji.201141890.

345. Smith, F.L., Savage, H.P., Luo, Z., Tipton, C.M., Lee, F.E., Apostol, A.C., Beaudin, A.E., Lopez, D.A., Jensen, I., Keller, S., and Baumgarth, N. (2023). *B-1 plasma cells require non-cognate CD4 T cell help to generate a unique repertoire of natural IgM*. J Exp Med ***220***. 10.1084/jem.20220195.

346. Savage, H.P., Yenson, V.M., Sawhney, S.S., Mousseau, B.J., Lund, F.E., and Baumgarth, N. (2017). *Blimp-1-dependent and -independent natural antibody production by B-1 and B-1-derived plasma cells*. J Exp Med ***214***, 2777-2794. 10.1084/jem.20161122.

Appendix

Figures

Figure 1: Hematopoiesis in the adult bone marrow.. 4

Figure 2: B cell development. 7

Figure 3: T cell-dependent generation of high-affinity, class-switched antibodies during the GC reaction. 9

Figure 4: Life cycle and transmission of *T. gondii*. 12

Figure 5: Lytic cycle of *T. gondii*. 12

Figure 6: Detection of *T. gondii*-derived molecules and cellular sources of IFNγ. 13

Figure 7: The LTβR, its ligands, and the lymphotoxin signaling network. 18

Figure 8: Canonical and alternative NF-κB signaling pathways. 20

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
Ag	Antigen
AID	Activation-induced cytidine deaminase
APC	Antigen presenting cell
BCR	B cell receptor
BM	Bone marrow
B_{reg}	Regulatory B cell
CCR	C-C-motif chemokine receptor
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CLP	Common lymphoid progenitor
CLR	C-type lectin receptor
CMP	Common myeloid progenitor
Cox	Cyclooxygenase
CRD	Cysteine-rich domain
CSR	Class-switch recombination
CTL	Cytotoxic T lymphocyte
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DC	Dendritic cell
DZ	Dark zone
FCS	Fetal calf serum

FO	Follicular helper T cell
GALT	Gut-associated lymphatic tissue
GAS	Gamma interferon activation site
GBP	Guanylate-binding protein
GC	Germinal center
GMP	Granulocyte-macrophage progenitor
GRA	Dense granule protein
GTP	Guanosine triphosphate
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
i.p.	Intraperitoneal
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IKK	Inhibitor of nuclear factor kappa-B kinase
IL	Interleukin
ILC	Innate lymphoid cell
iNOS	Inducible nitric oxide synthase
IRF	Interferon-regulatory factor
IRG	Immunity-related GTPase
ISG	Interferon-stimulated gene
ISRE	Interferon-stimulated response element
JAK	Janus activated kinase
JNK	c-Jun N-terminal kinase
LIGHT	Lymphotoxin-like, exhibits inducible expression, and competes with HSV glyco-protein D for herpesvirus entry mediator, a receptor expressed by T lymphocytes
LMPP	Lymphoid-primed multipotent progenitor
LN	Lymph node
LT	Lymphotoxin
LTβR	Lymphotoxin beta receptor
LZ	Light zone
MCMV	Murine Cytomegalovirus
MEP	Megakaryocyte-erythrocyte progenitor
mGBP	Murine guanylate-binding protein
MHC	Major histocompatibility complex
MHCI / II	Major histocompatibility complex class 1 / class 2
MLN	Mesenteric lymph node
moDC	Monocyte-derived dendritic cell
MPP	Multipotent progenitor
MZ	Marginal zone
Nab	Natural antibody
NEMO	NF-kappa-B essential modulator
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NIK	NF-κB-inducing kinase
NK cell	Natural killer cell

NKT	Natural killer T cell
NRL	Nod-like receptor
NuRD	Nucleosome remodeling and deacetylase
p.i.	Post-infection
PAMP	Pathogen-associated molecular pattern
PC	Plasma cell
pDC	Plasmacytoid dendritic cell
PerC	Peritoneal cavity
PP	Peyer's Patch
PRR	Pathogen recognition receptor
PV	Parasitophorous vacuole
RAG1/2	Recombination-activating genes 1 and 2
RBC	Red blood cell
ROP	Rhoptry protein
ROS	Reactive oxygen species
RT	Room temperature
S1P	Sphingosine-1-phosphate
SDF-1	Stromal cell derived factor 1 (*alias* CXCL12)
SHM	Somatic hypermutation
SLO	Secondary lymphoid organ
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TD	T cell-dependent
T_{FH}	Follicular helper T cell
TgCyp18	Toxoplasma gondii cyclophilin-18
TgIST	Toxoplasma gondii inhibitor of STAT1 transcription
Th cell	T helper cell
TI	T cell-independent
TIM	TRAF-interacting motifs
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TNFRSF	Tumor necrosis factor receptor superfamily
TRAF	TNFR-associated factor
T_{reg}	Regulatory T cell
V(D)J recombination	Variable, diversity, and joining element recombination
WT	Wild-type

Statutory Declaration / Eidesstattliche Erklärung

Statutory Declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources. Furthermore, the dissertation has not been submitted to any other faculty, nor has there been another unsuccessful or successful doctoral attempt.

Eidesstattliche Erklärung

Ich versichere an Eides statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter Wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf" erstellt worden ist. Darüber hinaus ist die Dissertation keiner anderen Fakultät vorgelegt worden, noch hat es einen weiteren erfolglosen oder erfolgreichen Promotionsversuch gegeben.

____

Marcel Helle

Düsseldorf, den 20.02.2025

Acknowledgments

Clearly, this doctoral thesis would not have been possible without the support of colleagues, friends, and family.

First and foremost, I would like to thank my first supervisor, Klaus Pfeffer, for giving me the opportunity to conduct research in his institute and for the freedom to explore my scientific curiosities. Thank you for your valuable support and feedback during our meetings, as well as for your efforts to maintain a great environment at the institute, even during challenging times.

I would also like to thank my second supervisor, Johannes Hegemann, whose questions helped me gain new perspectives on my research.

I would like to thank the Jürgen Manchot Foundation for funding my project as part of the Graduate School "Molecules of Infection IV (MOI IV)" and for providing valuable training and networking opportunities, and all my MOI colleagues for the great time we shared.

I want to thank my two supervisors, Uschi and Daniel, who helped shape this thesis. A special thanks goes to Uschi, whose unwavering support and commitment, both inside and outside the lab, were extraordinary – especially regarding all things mouse-related. Your detailed corrections and feedback have not only shaped this thesis but also made me a better scientist. I am truly grateful to have had you as my supervisor.

I also want to thank the entire Pfeffer group and all other colleagues at the institute. Special thanks to Julia, Nicole, and Sonja, for your support with the mice. The data presented in this thesis would not have been possible without your help, and I'm sure you also saved me from losing a few fingers in the process.

Right in the middle of things, just as she is known for, I want to thank Veroniça. You have been a great friend over the years and an excellent travelling companion to Sardinia and Strasbourg. This PhD would have been far less fun without you, and I'm happy we shared this journey.

I want to thank Marcel Marson. You were a fantastic roommate, especially during our trips and MOI events, as well as during the time we shared our downstairs office. As they say, "It's always good to have a Marcel by your side!" Thank you for the good times.

The same goes for Sebastian. We connected in the early days of my Master's and shared the ride all the way to the end of my PhD. Thank you for this amazing time!

I also want to thank Flaminia, Nik R. Komischke, and Lars. It wouldn't have been the same without you, and I'm grateful you were a part of my journey!

I look forward to reuniting with all of you guys in the future, perhaps over a glass of wine – now that it's allowed!

Last but not least, I would like to thank Anja, Julia, and my parents for their continuous support and for helping me clear my head when I needed it most. This would not have been possible without you, and you are the best!

Cheers!