Analysis of the lymphotoxin β receptor in immune responses against *Toxoplasma gondii*

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Anne Tersteegen
from Essen

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

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Supervisor: Univ.-Prof. Dr. Klaus Pfeffer

Co-supervisor: Prof. Dr. Johannes Hegemann

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Abstract

Lymphotoxin β receptor (LTβR) mediated signaling plays an important role in the initiation of immune responses to a broad range of pathogens, including the obligate intracellular parasite *Toxoplasma gondii* (*T. gondii*). *T. gondii* resides and replicates in a parasitophorous vacuole (PV), which protects the parasite from host defence. Infection with the parasite causes toxoplasmosis; globally, nearly one-third of the world's human population is infected which constitutes a serious health problem. Toxoplasmosis usually causes no or only mild flu-like symptoms, but immunocompromised patients can be severely affected. Also, in a primary infection during pregnancy, the parasite can cross the placental barrier, leading to miscarriage or severe birth defects in the child.

In mice, LTβR signaling is crucial for surviving acute toxoplasmosis. Compared to wildtype (WT) animals, LTβR-deficient ($LT\beta R^{-/-}$) mice show a markedly increased mortality in a *T. gondii* infection model: About 90-100 % of the animals succumb to the infection. However, little is known about the mechanisms responsible for the detrimental loss of immunity in the absence of the LTβR. Therefore, in the present thesis immune responses, especially cytokine production, the presence of anti-parasitic effector molecules as well as T- and B cell responses were investigated in an *in vivo T. gondii* infection model in LTβR-deficient mice.

This thesis shows that LTβR deficiency dysregulates multiple arms of innate and adaptive immune responses in a *T. gondii* infection. Infected $LT\beta R^{-/-}$ mice show altered interferon gamma (IFNγ) regulation in lungs, spleen and brain, reduced IFNγ-controlled host effector molecule expression, altered frequencies of immune cell populations and impaired T cell differentiation/functionality. Furthermore, infected $LT\beta R^{-/-}$ mice lack an anti-parasite specific immunoglobulin (Ig) G response which leads to the loss of immune control. Reconstitution with toxoplasma immune serum significantly prolongs the survival of *T. gondii* infected $LT\beta R^{-/-}$ mice, demonstrating an important novel role for LTβR signaling in B cell mediated immune responses to *T. gondii*. Ultimately, due to these defects, $LT\beta R^{-/-}$ mice are not able to control the parasite and succumb to the infection. In addition, transcriptomics suggests a possible and as yet unreported suppression of the B cell response by *T. gondii* in WT mice.

These results provide new insights into the role of the LTβR in *T. gondii* infection and could provide valuable information for further studies exploring new therapeutic strategies, especially sorely needed vaccines for human toxoplasmosis.

Zusammenfassung

Die Lymphotoxin β Rezeptor (LTβR) vermittelte Signalübertragung spielt eine große Rolle in der Generierung von Immunantworten gegen eine Vielzahl von Pathogenen. Einer dieser Pathogene ist der obligat intrazelluläre Parasit *Toxoplasma gondii* (*T. gondii*), welcher innerhalb einer parasitophoren Vakuole (PV) repliziert, die den Parasiten vor der Immunantwort des Wirtes schützt. Eine Infektion mit dem Parasiten führt zur sogenannten Toxoplasmose. Weltweit sind ungefähr ein Drittel der Menschen infiziert, was ein ernst zu nehmendes Gesundheitsproblem darstellt. In gesunden Individuen äußert sich eine Toxoplasmose lediglich in leichten Erkältungssymptomen. In immunkompromittierten Individuen kann eine Toxoplasmose jedoch zu schwerwiegenden Komplikationen führen. Bei einer Erstinfektion in der Schwangerschaft kann der Parasit die Plazentaschranke durchqueren, was zu Fehlgeburten oder schweren Geburtsfehlern des Kindes führen kann.

In Mäusen ist die LTβR Signalübertragung essentiell zum Überleben einer akuten Toxoplasmose. LTβR-defiziente ($LT\beta R^{-/-}$) Mäuse weisen eine deutlich erhöhte Mortalität gegenüber Wildtyp (WT) Mäusen auf. Circa 90-100% der Tiere versterben an der Infektion. Bisher ist jedoch sehr wenig über die Mechanismen, die für diesen gravierenden Verlust der Immunität verantwortlich sind, bekannt. Deshalb wurden in der vorliegenden Arbeit die Immunantwort, insbesondere die Zytokinproduktion, das Vorhandensein anti-parasitärer Effektor Moleküle, und die T- und B-Zell Antwort im Rahmen eines *in vivo T. gondii* Infektionsmodelles in LTβR-defizienten Mäusen analysiert.

Diese Studie zeigt, dass das Fehlen des LTβR im Rahmen des *T. gondii* Infektionsmodelles verschiedene Arme der angeborenen und erworbenen Immunantwort dereguliert. Infizierte $LT\beta R^{-/-}$ Mäuse zeigen eine veränderte Interferon gamma (IFNγ) Regulation in Lunge, Milz und im Gehirn, eine verringerte Expression von IFNγ induzierten Effektor Molekülen, veränderte Immunzellpopulationen und eine beeinträchtigte Differenzierung und Funktionalität von T-Zellen auf. Des Weiteren fehlt diesen Mäusen eine spezifische anti-parasitäre immunglobulin (Ig) G Antwort, wodurch die Immunkontrolle nicht mehr gewährleistet ist. Eine Rekonstitution mit Toxoplasma Immunserum konnte das Überleben von $LT\beta R^{-/-}$ Mäusen signifikant verlängern, was auf eine wichtige, bisher noch nicht beschriebene Rolle des LTβR in der B Zell vermittelten Immunantwort auf *T. gondii* hinweist. Letztendlich sind $LT\beta R^{-/-}$ Mäuse aufgrund dieser Defekte nicht in der Lage, das Parasitenwachstum zu kontrollieren und erliegen der Infektion. Darüber hinaus deuten Transkriptomdaten auf eine mögliche *T. gondii* vermittelte Suppression der B Zell Antwort in WT Tieren hin.

Diese Ergebnisse liefern neue Einblicke in die Rolle des LTβR in einer *T. gondii* Infektion und liefern wertvolle Hinweise für weitere Studien zur Erforschung neuer therapeutische Strategien, insbesondere dringend benötigter Vakzine für die humane Toxoplasmose.

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1. Introduction

1.1 *Toxoplasma gondii* (*T. gondii*)

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan that can infect all nucleated cells of warm blooded animals, including humans, leading to the disease toxoplasmosis (Dubey, Lindsay et al. 1998, Flegr, Prandota et al. 2014). Based on their phenotypes in mice, which differ in growth, migration, transmigration and virulence, *T. gondii* strains have been classified into three sub-types (Sibley and Boothroyd 1992, Howe and Sibley 1995, Grigg, Ganatra et al. 2001, de Moura, Bahia-Oliveira et al. 2006). Type I strains are highly virulent causing lethal infections, whereas type II and in particular type III strains show only low virulence in mice (Barragan and Sibley 2003). Several studies showed that type II strains (e.g. ME49) are the most prevalent and frequently found in human toxoplasmosis (Sibley and Boothroyd 1992, Howe and Sibley 1995, Howe, Honore et al. 1997, Schluter, Daubener et al. 2014).

1.1.1 Life cycle

The life cycle of *T. gondii* (Figure 1) encompasses two reproductive stages: the sexual stage in definitive and the asexual stage in intermediate hosts. Definitive hosts are felines (Feloidea) where *T. gondii* can sexually reproduce only in the epithelium of the digestive tract. Intermediate hosts are all warm-blooded animals, including humans and livestock, where asexual reproduction takes places. Infection of humans can occur through oral uptake of cysts (e.g. raw/undercooked meat from infected intermediate hosts) or oocysts that are contained in cat feces and contaminated vegetables, water, soil and, of course, cat litter. Ingested oocysts and tissue cysts release sporozoites and bradyzoites, respectively, which invade the epithelium of the digestive tract where they differentiate into tachyzoites (Dubey, Lindsay et al. 1998). *T. gondii* resides and replicates within infected cells in a specialized membranous compartment called the parasitophorous vacuole (PV) which is derived from the host plasma membrane. During the acute phase of infection, *T. gondii* undergoes a fast lytic cycle (Figure 2) where infectious tachyzoites spread via the blood stream and lymph. If the PV is disrupted *T. gondii* is unable to survive within host cells. Under pressure of the host immune response, tachyzoites eventually convert to metabolically less active bradyzoites. These reside in tissue cysts in the central nervous system and muscle where they are able to evade the immune system, establishing latent toxoplasmosis (Hartmann 2016).

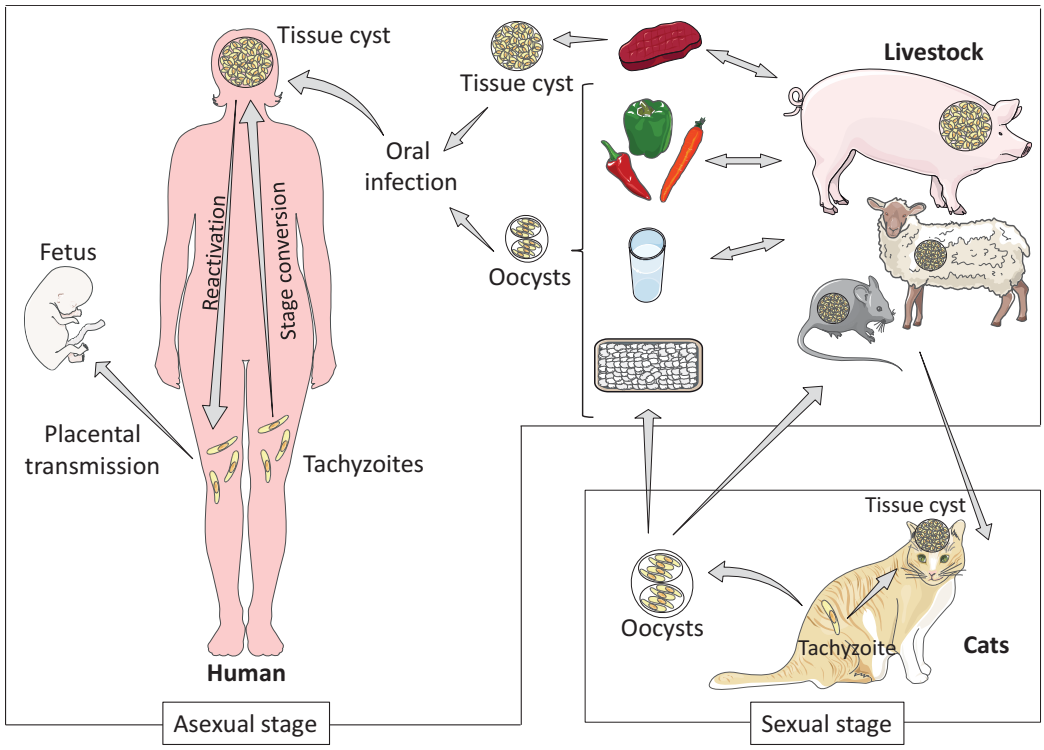


Figure 1: Life cycle of *T. gondii*. Infected feloidea (e.g. cats) shed oocysts via their feces into the environment. These oocysts can contaminate water, soil and vegetables and can be taken up by intermediate hosts (e.g. livestock) where sporozoites ultimately develop into tissue cysts. Oocysts from contaminated products as well as tissue cysts from undercooked meat can easily be consumed by humans. Under pressure of the host immune response infective tachyzoites can convert to bradyzoites that reside in tissue cysts. Vice versa, in immunocompromised individuals bradyzoites can reactivate into tachyzoites. In pregnant women tachyzoites are able to cross the placental barrier and can infect the fetus. The graphic was designed with Servier Medical Art License (http://creativecommons.org/licenses/by/3.0/). Changes were made to the provided elements.

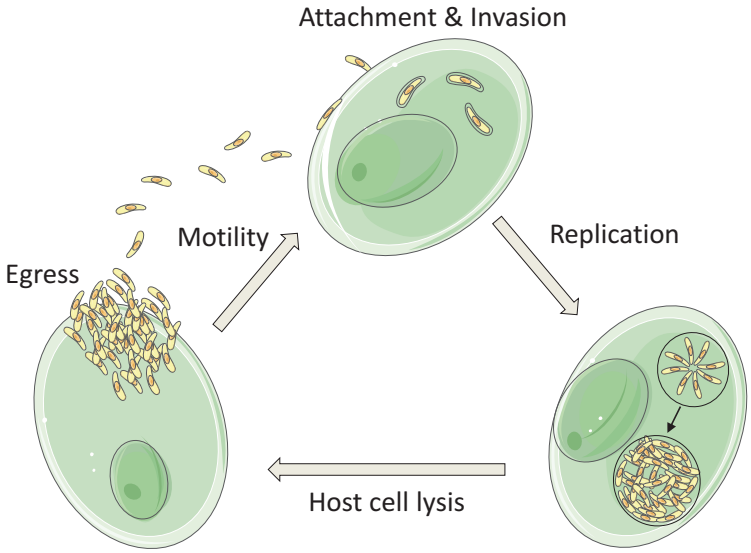


Figure 2: Lytic cycle of *T. gondii*. During acute infection infectious *T. gondii* tachyzoites invade host cells and replicate in parasitophorous vacuoles, resulting in host cell lysis. Motile tachyzoites egress from the lysed cell and then infect surrounding cells, thus continuing the lytic cycle. The graphic was designed with Servier Medical Art License (http://creativecommons.org/licenses/by/3.0/). Changes were made to the provided elements.

1.1.2 Toxoplasmosis

Toxoplasmosis is a global infectious disease caused by *T. gondii*. At least one third of the human population is infected, making *T. gondii* one of the most successful parasites (Halonen and Weiss 2013). The prevalence of human toxoplasmosis varies in different parts of the world (up to 75%) (Pappas, Roussos et al. 2009), correlating positively with age (Montoya and Liesenfeld 2004), warm and humid climates (Bojar and Szymanska 2010), low quality of water resources and hygiene (Jones and Dubey 2010) as well as prevalent consumption of undercooked meat (Bojar and Szymanska 2010). In immunocompetent individuals primary/acute toxoplasmosis is usually benign but may cause mild flu-like symptoms or lymphadenopathy, and the immune response is able to contain the chronic stage of infection. In immunocompromised individuals, e.g. acquired immune deficiency syndrome (AIDS) patients, cancer patients undergoing chemotherapy, or as a consequence of immunosuppressive therapy after transplantation, a primary infection as well as the reactivation of a latent toxoplasmosis can lead to life-threatening conditions (Howard, Hunn et al. 2011). While a primary infection is mostly asymptomatic in pregnant women, tachyzoites are able to cross the placental barrier and infect the fetus. This can lead to congenital toxoplasmosis or even to miscarriage and stillbirth (Montoya and Liesenfeld 2004, Saadatnia and Golkar 2012). In addition, there is increasing, albeit controversial, evidence that chronic infection could contribute to psychiatric and neurodegenerative disorders (Donley, Olson et al. 2016).

1.1.3 Immune response against *T. gondii*

Innate immunity acts as a first line of host defence in the containment of a *T. gondii* infection. Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), Nod-like receptors (NLRs), and C-type lectins, detect pathogen-associated molecular patterns (PAMPs) and ensure both intrinsic and extrinsic recognition of invading pathogens (Beutler, Jiang et al. 2006, Sasai and Yamamoto 2013). Once *T. gondii* tachyzoites egress from the primarily infected cell, PAMPs, primarily profilin, are recognized by antigen presenting cells (APCs) (Figure 3). These APCs, e.g. dendritic cells (DCs), produce inflammatory cytokines like interleukin 12 (IL-12) and tumor necrosis factor alpha (TNFα) which are important for the activation of the innate immune response and the subsequent adaptive immune response. Early in infection, interferon gamma (IFNγ) production is vital for inducing cell autonomous immune responses, resulting in increased expression of proteins that are essential for parasite containment and later for the maintenance of the chronic stage of infection (Gazzinelli, Hieny et al. 1993, Hunter, Subauste et al. 1994, Yarovinsky 2014).

However, the mechanism by which *T. gondii* activates these cell types as well as how activation leads to the induction of acquired immune responses is still not fully understood.

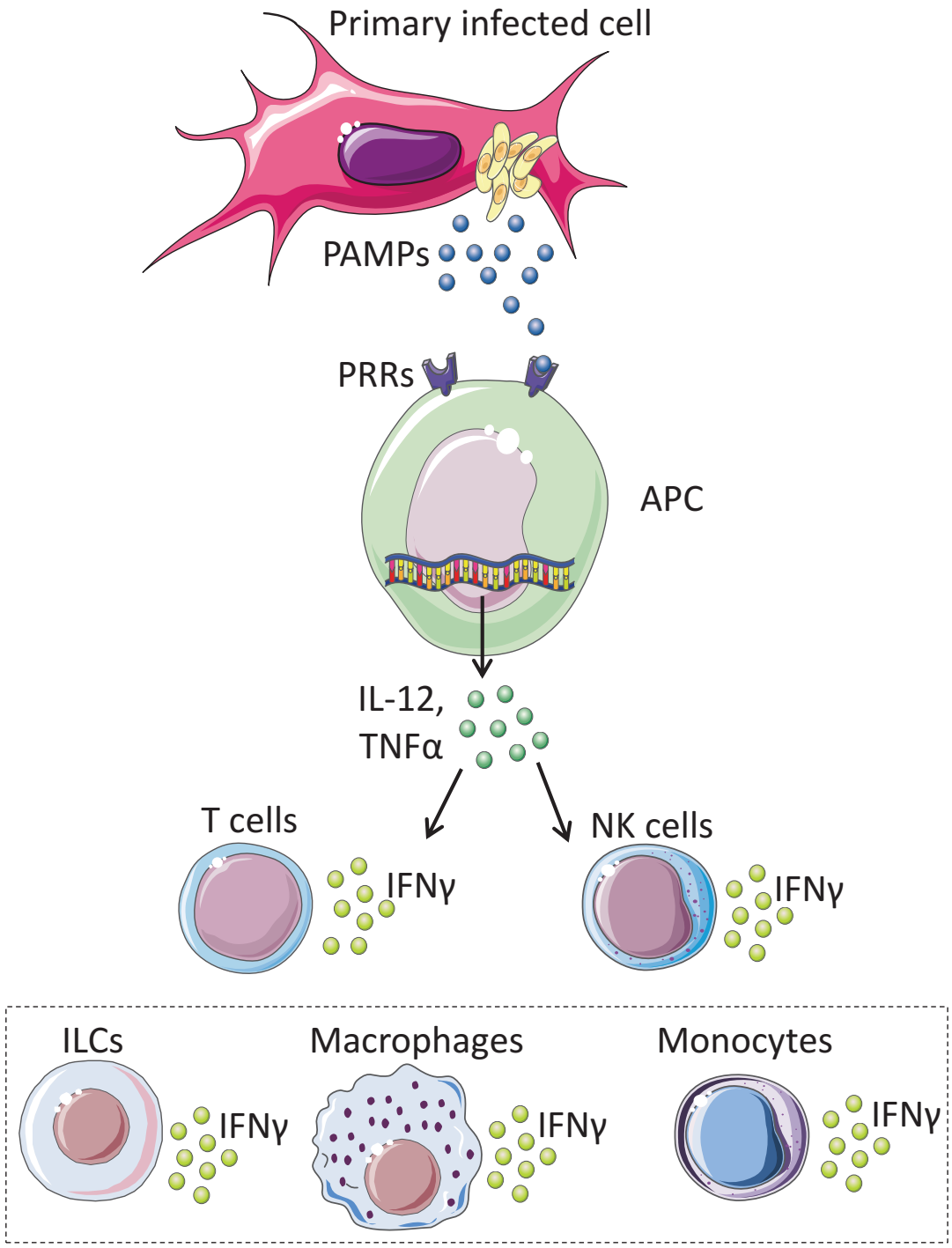


Figure 3: Innate immune response to *T. gondii*. *T. gondii* tachyzoites egress from a primarily infected cell and pathogen-associated molecular patterns (PAMPs) are recognized by antigen presenting cells (APCs) via pattern recognition receptors (PRRs). These APCs produce proinflammatory cytokines such as interleukin 12 (IL-12) and interferon gamma (IFNγ) that in turn lead to stimulation of natural killer (NK) and T-cell proliferation. Besides T- and NK cells innate lymphoid cells (ILCs), macrophages, and monocytes are also able to secrete IFNγ. The graphic was designed with Servier Medical Art License (http://creativecommons.org/licenses/by/3.0/). Changes were made to the provided elements.

1.2 Interferon gamma (IFNγ)

Interferons (IFNs) as powerful signaling and regulatory molecules of the immune system possess potent pleiotropic effects in antimicrobial, antitumor and immunomodulatory processes by regulating the expression of hundreds of genes (Gresser 1990, Boehm, Klamp et al. 1997, Pestka, Krause et al. 2004, MacMicking 2012). IFNγ, the only type II interferon, is a cytokine that has been shown to be crucial for both innate and adaptive immunity against various pathogens, including parasites. IFNγ is typically produced by T cells (Scharton-Kersten, Nakajima et al. 1998, Denkers 1999) and NK cells (Gaddi and Yap 2007). Whether other cell populations are also capable of producing interferon gamma has not yet been fully clarified as the available data are inconclusive. However, studies on the single cell level demonstrated that also ILCs (Wagage, Harms Pritchard et al. 2015, Ivanova, Denton et al. 2019), DCs (Fricke, Mitchell et al. 2006), macrophages (Darwich, Coma et al. 2009), and monocytes (Kraaij, Vereyken et al. 2014) can produce IFNγ. Besides being produced by immune cells, IFNγ is also involved in the activation and differentiation of T cells, B cells, macrophages, NK cells as well as other non-immune cells such as fibroblasts and endothelial cells. A variety of cellular processes are coordinated by IFNγ-induced transcriptional regulation of immune relevant genes via Janus Kinase (JAK)/signal transducer and activator of transcription (STAT) signaling (O'Shea, Gadina et al. 2002).

1.2.1 The IFNγ signaling pathway

The IFNγ receptor (IFNγR) consists of two subunits: the ligand binding IFNγR1 and the signal transmitting IFNγR2 which mediate signal transduction via the JAK/STAT pathway (Figure 4). IFNγ binding leads to receptor dimerization that in turn induces JAK1 and JAK2 activation and subsequent phosphorylation of a tyrosine residue (Tyr-440) in the intracellular domain of the IFNγR1, resulting in STAT1 recruitment, phosphorylation (pSTAT1) and homodimer assembly (Greenlund, Farrar et al. 1994, Kisseleva, Bhattacharya et al. 2002). These homodimers, also known as gamma-interferon activation factors (GAFs), then translocate into the nucleus where they bind to gamma-interferon activated sites (GAS), thereby activating the expression of primary response genes (Levy, Lew et al. 1990, Decker, Lew et al. 1991). Among these genes are guanylate-binding proteins (GBPs), indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase *(*iNOS) as well as transcription factors such as interferon-regulatory factor 1 (IRF-1) (Degrandi, Konermann et al. 2007, MacKenzie, Heseler et al. 2007, Yarovinsky 2014, Bogdan 2015). After signal transmission, receptor chains are internalized, they dissociate and are transported to the cell membrane for recycling. While IFNγ signal transduction is mainly associated with pSTAT1 homodimers, it can also activate the expression of other family members such as STAT3 or STAT5, leading to the formation of heterodimers that induce

different subsets of genes. Vice versa, type I and type III IFN signaling is also able to induce pSTAT1 homodimer formation (Takaoka, Mitani et al. 2000, Pestka, Krause et al. 2004). In different autoimmune diseases such as rheumatoid arthritis, that is mostly TNF-mediated (Feldmann and Maini 2008), dysregulated expression of IFNγ could be identified (Nielen, van Schaardenburg et al. 2004, Schurgers, Billiau et al. 2011, Kim and Moudgil 2017) and is associated with disease progression (Lee, Kwon et al. 2017). Further, IFNγ is described to influence the tumor microenvironment (Le Poole, Riker et al. 2002), demonstrating the need for tight IFNγ regulation. This tight control is mediated via negative regulators of the JAK/STAT pathway with suppressor of cytokine signaling (SOCS) proteins being the most prominent representatives. SOCS expression is induced by IRF-1, establishing a negative feedback loop to control IFNγ signaling (Endo, Masuhara et al. 1997, Starr, Willson et al. 1997).

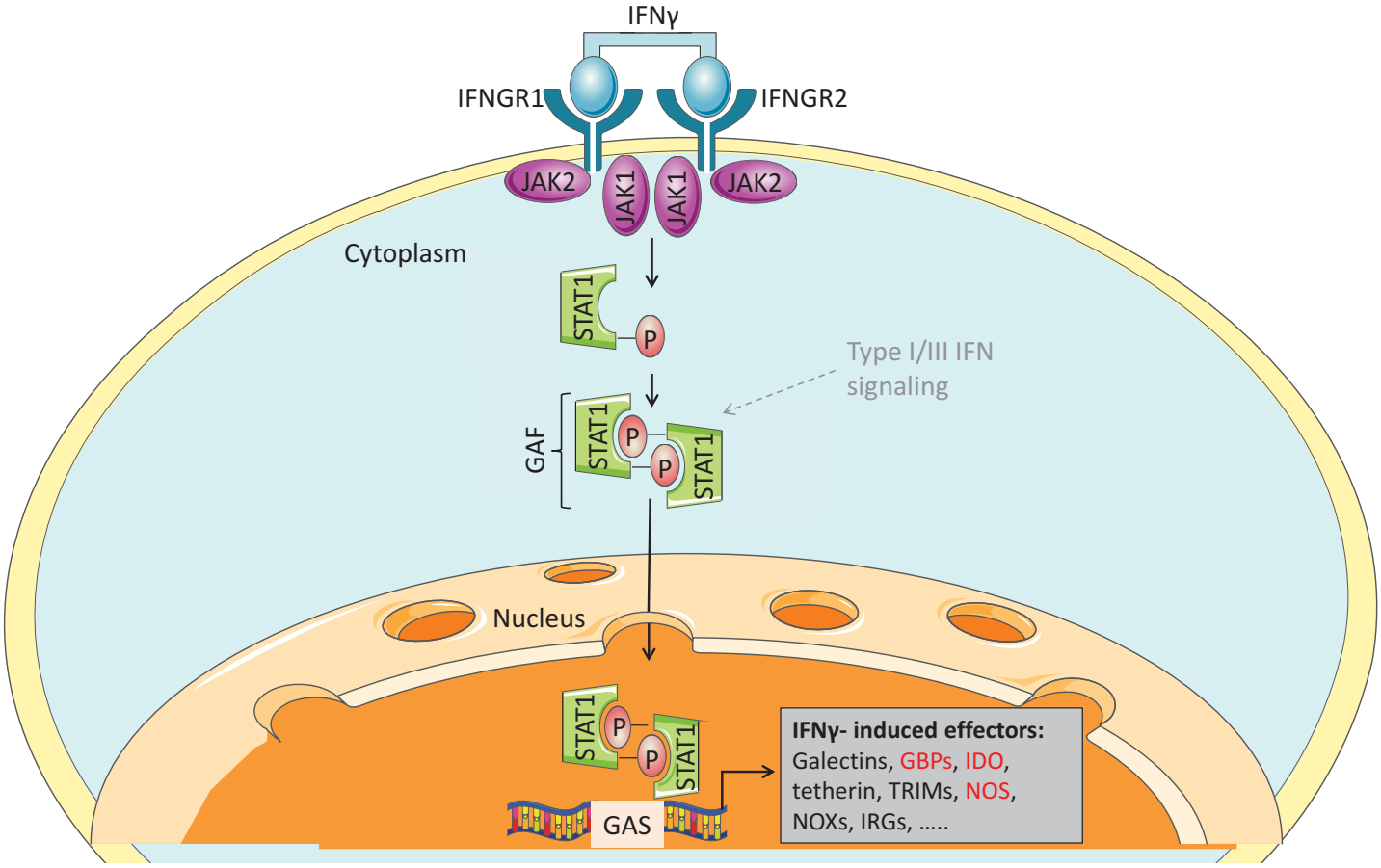


Figure 4: The IFNγ signaling pathway. The IFNγ receptor (IFNGR) consists of two distinct subunits, the IFNGR1 and IFNGR2, which engage IFNγ. Signaling occurs via Janus kinase (JAK)-mediated phosphorylation and dimerization of signal transducers and activators of transcription 1 (STAT1) proteins. These phosphorylated (p)STAT1 homodimers, also known as gamma-interferon-activation factors (GAFs), are primarily but not exclusively associated with IFNγ signaling and can also be induced by type I or type III IFN signaling. GAFs then translocate into the nucleus where they bind to gamma-interferon activation sites (GAS) inducing effector gene expression. The graphic was designed with Servier Medical Art License (http://creativecommons.org/licenses/by/3.0/). Changes were made to the provided elements.

1.2.2 Role of IFNγ-induced effector molecules in *T. gondii* infection

Induction of IFNγ-mediated effector molecules (Figure 5) is known to play a vital role in *T. gondii* infection due to their anti-parasitic effector functions. Expression of IDO (Pfefferkorn, Rebhun et al. 1986, Murray, Szuro-Sudol et al. 1989) and iNOS (Scharton-Kersten, Yap et al. 1997) are important for restricting parasite replication. Murine GBPs (mGBPs) are known to be essential for parasite containment (Degrandi, Konermann et al. 2007, Yamamoto, Okuyama et al. 2012, Steffens, Beuter-Gunia et al. 2020) as they contribute to the disruption of the PV as well as the *T. gondii* cell membrane. All of these IFNγ induced effector mechanisms contribute to *T. gondii* containment, but the extent to which cells use these different mechanisms is dependent on the cell type (Yarovinsky 2014).

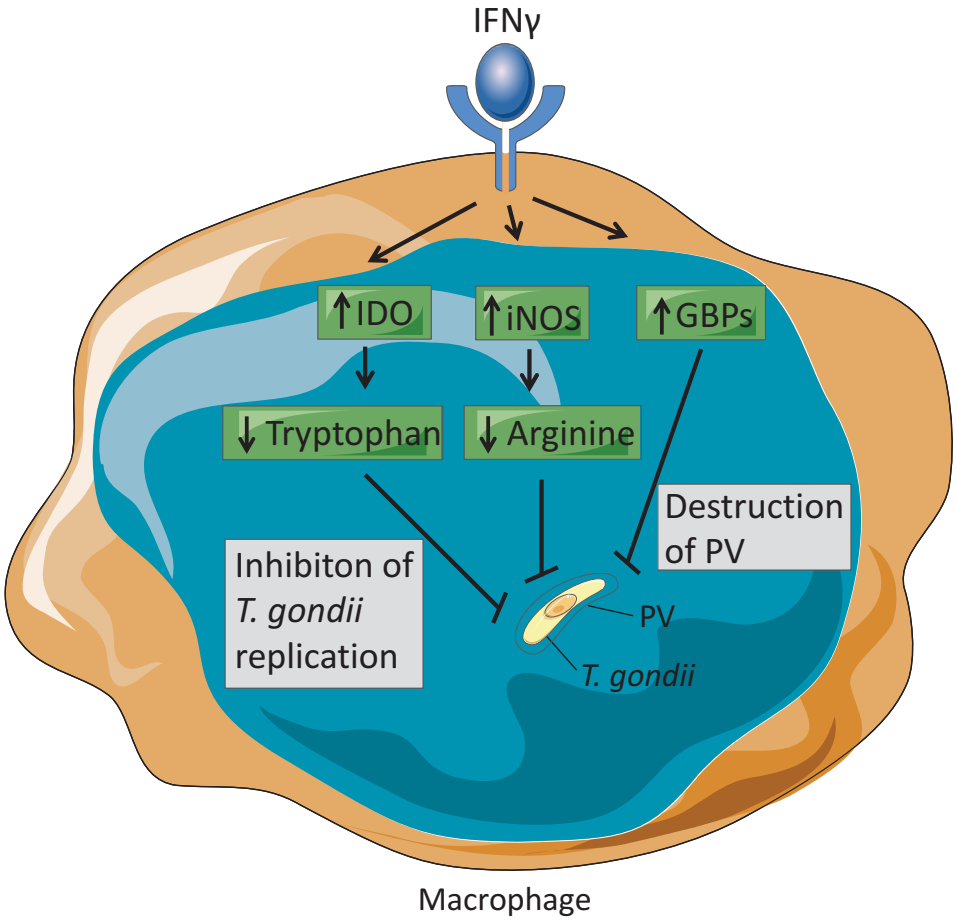


Figure 5: IFNγ-induced effector molecules in host cell defence against *T. gondii*. IFNγ signaling results in the induction of expression of effector molecules such as indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase (iNOS) and guanylate-binding proteins (GBPs), all capable of exerting anti-parasitic effects. Production of metabolites from both, IDO and iNOS, inhibit *T. gondii* replication via depletion of essential amino acids such as tryptophan or arginine. GBPs target the parasitophorous vacuole (PV), leading to the destruction of the PV and the parasite within. Induction of IFNγ-induced effector mechanism contributes to *T. gondii* containment; however expression of the specific molecules differs depending on the cell type. The graphic was designed with Servier Medical Art License (http://creativecommons.org/licenses/by/3.0/). Changes were made to the provided elements.

1.2.2.1 Indoleamine 2,3-dioxygenase (IDO)

IDO mediates an antiproliferative effect on *T. gondii* via conversion of tryptophan into *N*-formylkynurenine. This conversion leads to a depletion of the essential amino acid tryptophan resulting in cell starvation, because *T. gondii* is tryptophan auxotroph and therefore vulnerable to this host defence mechanism (Figure 5) (Pfefferkorn 1984, Pfefferkorn, Rebhun et al. 1986). An effective role of IDO was shown in various *T. gondii* infected human cell lines such as fibroblasts (Pfefferkorn 1984), epithelial, and endothelial cells (MacKenzie, Heseler et al. 2007). However, the anti-parasitic role of IDO could not be confirmed in murine macrophages and mesenchymal stromal cells (Schwartzman, Gonias et al. 1990, Meisel, Brockers et al. 2011). Moreover, susceptibility of IDO1 deficient mice was not affected in an acute *T. gondii* infection, indicating it is ineffective as an anti-parasitic defence mechanism in mice (Ufermann, Domrose et al. 2019).

1.2.2.2 Inducible nitric oxide synthase (iNOS)

iNOS does not only lead to the production of nitric oxide (NO), which has microbicidal activities, but also leads to the depletion of arginine during NO synthesis. Since *T. gondii* is strictly arginine auxotroph, the arginine deprivation blocks *T. gondii* replication (Figure 5) *(Fox, Gigley et al. 2004)*. iNOS deficient mice revealed a more complex role for NO-mediated host resistance to *T. gondii in vivo*, as they control parasite replication during the acute stage of infection, but show increased susceptibility during the chronic stage of infection *(Scharton-Kersten, Yap et al. 1997)*. This indicates that iNOS is not ultimately required in acute infection for tachyzoite elimination, but its involvement in the chronic stage suggests that key effector mechanisms might differ between the acute and the chronic stage of *T. gondii* infection *(Yap and Sher 1999)*.

1.2.2.3 Murine guanylate-binding proteins (mGBPs)

mGBPs are interferon induced GTPases essential for host defence against intracellular pathogens such as *T. gondii* (Degrandi, Konermann et al. 2007). Seven human and 13 murine GBPs have been described so far, the latter being located in two genomic clusters on chromosomes 3 and 5 (Olszewski, Gray et al. 2006, Kresse, Konermann et al. 2008). Studies showed that mGBP2, and probably other mGBPs, are able to form homo- and heterooligomers and cluster in the cytoplasm in vesicle-like structures (VLS) (Vestal, Gorbacheva et al. 2000, Kravets, Degrandi et al. 2016). Resistance to *T. gondii* seems to be mediated by accumulation of GBPs at the PV membrane of the parasite which destabilizes vacuolar integrity (Figure 5)

(Degrandi, Konermann et al. 2007, Selleck, Fentress et al. 2013, Kravets, Degrandi et al. 2016). Recruitment of the different mGBPs towards the *T. gondii* PV follows a specific hierarchical sequence and order (Kravets, Degrandi et al. 2016, Steffens, Beuter-Gunia et al. 2020). Ultimately, different mGBPs act in concert to destroy the PV and the parasite within (Degrandi, Konermann et al. 2007, Kim, Shenoy et al. 2012, Yamamoto, Okuyama et al. 2012, Degrandi, Kravets et al. 2013), thus mediating major functions in parasite elimination. *In vivo*, high susceptibility of *T. gondii* infected mGBP2 deficient and mGBP7 deficient mice could be shown, further underlining the crucial role of mGBPs in acute *T. gondii* infection (Degrandi, Kravets et al. 2013, Steffens, Beuter-Gunia et al. 2020).

1.3 The tumor necrosis factor (TNF) / TNF receptor (TNFR) superfamily

The tumor necrosis factor (TNF) / TNF receptor (TNFR) superfamily consists of more than 40 identified ligands and receptors that share structural properties but have distinct functions. They create a key communication network which is involved in diverse mechanisms in immune responses and beyond. The family members, expressed by a wide range of cell types, fulfil multiple, sometimes contradictory roles: in inflammation and host defence, organogenesis of lymphoid organs, autoimmunity, and tumor immunotherapy as well as in cell differentiation, proliferation and apoptosis. Core members of this family include TNF and lymphotoxin (LT) as well as their corresponding receptors, which are described as potent and crucial cytokines (Locksley, Killeen et al. 2001, Hehlgans and Pfeffer 2005, Ward-Kavanagh, Lin et al. 2016, So and Ishii 2019).

1.3.1 Core members of the TNF/TNFR superfamily

The ligands TNF (TNFα), LTα (LTα_3), LTβ (LT$\alpha_1\beta_2$; LT$\alpha_2\beta_1$), LIGHT (homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for herpes virus entry mediator [HVEM], a receptor expressed by T lymphocytes), BTLA (B- and T lymphocyte attenuator) and DcR3 (decoy receptor 3) and their cognate receptors TNFRI (TNFR-p55), TNFRII (TNFR-p75), LTβ receptor (LTβR) and HVEM constitute the core members of the TNF/TNFR superfamily as illustrated in Figure 6 (Mauri, Ebner et al. 1998, Hehlgans and Pfeffer 2005). They form a complex signaling network in which BTLA and DcR3 play only a minor role for this thesis.

The receptors are characterized as type I transmembrane glycosylated proteins with an intracellular domain, a transmembrane domain (TMD) and cysteine-rich domains (CRDs) in

their extracellular domain. CRDs can be seen as the hallmark of the TNFR superfamily, they are highly conserved and their number varies between one to six, depending on the respective TNFR superfamily member (Hehlgans and Pfeffer 2005). CRDs mediate receptor specificity and affinity to the respective ligand(s) (Albarbar, Dunnill et al. 2015). The ligands are characterized as type II transmembrane proteins that contain an intracellular N-terminus and an extracellular C-terminus. Homotrimeric TNF and LIGHT can be either expressed on the cell membrane or shed via proteolytic cleavage releasing the soluble cytokine. LTα is a soluble protein only. Secreted LTα will assemble as a homotrimer ($LT\alpha_3$) or as a complex with membrane-bound LTβ to generate two distinct heterotrimers: $LT\alpha_1\beta_2$ and $LT\alpha_2\beta_1$ (Idriss and Naismith 2000, Bodmer, Schneider et al. 2002). One feature of the TNF/TNR superfamily is that one ligand can bind several receptors and that one receptor can bind several ligands (Hehlgans and Pfeffer 2005, Albarbar, Dunnill et al. 2015).

TNF is known as a critical factor in the induction of inflammatory events, and signals via two distinct receptors: TNFRI and TNFRII (Ware 2003, Walczak 2011). Both receptors can be engaged by $LT\alpha_3$ and $LT\alpha_2\beta_1$, but while TNFRI contains a TNFR-associated death domain (TRADD) and can bind soluble and membrane-bound TNF, TNFRII is mainly activated via membrane-bound TNF (Grell 1995).

HVEM is able to switch between proinflammatory and inhibitory signaling by acting as both ligand and receptor for multiple ligands and co-receptors thereby creating a complex communication network. Ligands of HVEM include $LT\alpha_3$, BTLA and LIGHT, the latter also binding to the LTβR (Kaye, Hirst et al. 2008, Murphy and Murphy 2010, Cheung and Ware 2011, Sedy, Bekiaris et al. 2014).

Signal transduction of the TNFR superfamily follows recruitment of TNFR-associated factor (TRAF) proteins over TRAF binding motifs, except for TNFRI which signals via TRADD. Six different TRAF proteins have been described as being involved, however, not every receptor employs every TRAF protein and recruitment of different TRAF proteins leads to distinct signaling outcomes. Recruitment of adaptor molecules mediates signal transduction and subsequent activation of canonical and alternative NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B cells) or JNK (c Jun N-terminal kinases) pathways which can ultimately lead to induction of cell death, apoptosis, but also to cell survival or inflammation. Notably, the signaling outcome is context dependent und differs due to the involved ligands, receptors, adaptors as well as the specific physiological and biological state of the cell (Dejardin, Droin et al. 2002, Remouchamps, Boutaffala et al. 2011, Albarbar, Dunnill et al. 2015, Mitchell, Vargas et al. 2016).

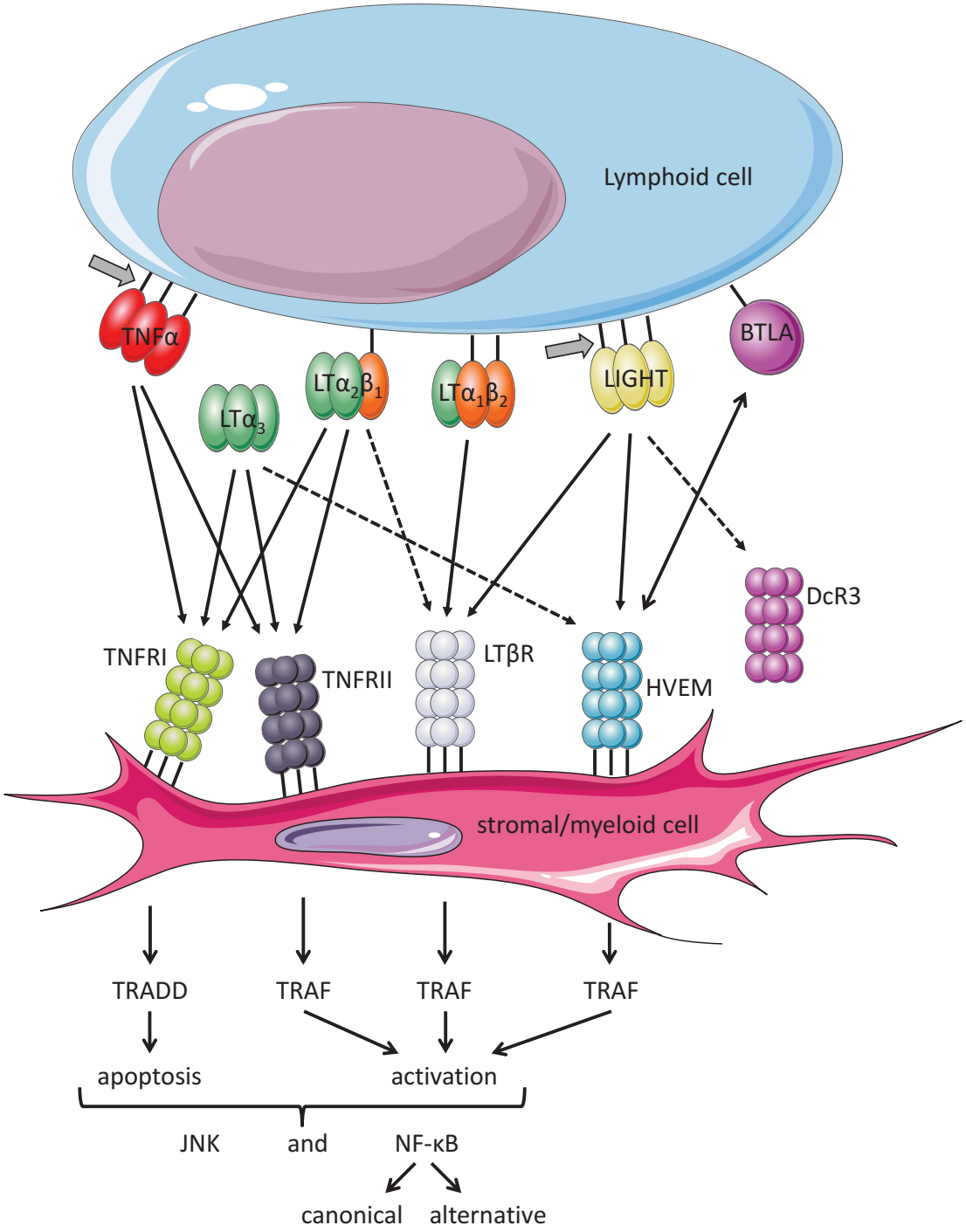


Figure 6: The lymphotoxin-related signaling network. The diagram illustrates the cross – utilization of ligands and receptors related to lymphotoxins. Arrows define high affinity binding; dotted lines indicate low affinity binding and arrowheads define signaling directionality, with dual arrowheads specifying bidirectional signaling. Grey arrows indicate cleavage sites. TNFα, $LT\alpha_3$, $LT\alpha_2\beta_1$, $LT\alpha_1\beta_2$, and LIGHT as TNF-related ligands are depicted as trimers expressed on lymphoid cells. Their cognate receptors, TNFRI, TNFRII, LTβR, and HVEM can be expressed on stromal and myeloid cells. Decoy receptor-3 (DcR3) when secreted binds to Fas ligand (not shown). Signal transduction occurs via recruitment of TRAF domains, except for the death domain containing TNFRI that also recruits TRADD for signal transduction, resulting in pathway activation or apoptosis. JNK and NF-κB pathways are the two main downstream signaling pathways. TNFRI can only signal via the canonical NF-κB pathway, whereas other receptors like the LTβR are able to signal via both the canonical and alternative NF-κB pathway with distinct outcomes. The graphic was designed with Servier Medical Art License (http://creativecommons.org/licenses/by/3.0/). Changes were made to the provided elements.

1.3.2 The lymphotoxin β receptor (LTβR)

The LTβR interacts with the two different LTα/LTβ heterotrimeric complexes and LIGHT as ligands, depicted in Figure 7 (Crowe, VanArsdale et al. 1994, Zhai, Guo et al. 1998). While $LT\alpha_1\beta_2$ is LTβR-specific, $LT\alpha_2\beta_1$ – of minor importance compared to $LT\alpha_1\beta_2$, but with distinct functions - binds to the LTβR only with low affinity (Androlewicz, Browning et al. 1992, Mauri, Ebner et al. 1998, Remouchamps, Boutaffala et al. 2011). Both need cell-cell contact for receptor activation because while the LTα chain is not membrane bound, the LTβ chain is and cannot be cleaved from the cell surface. Signal transduction via the LTβR occurs via TRAF adaptor proteins, TRAF-2, -3 or -5, that produce distinct effects on further downstream signaling pathways, thus regulating signaling outcome. Generally, LTβR activation mediates activation of NF-κB signaling via TRAF2 and TRAF5, but TRAF3 is conspicuously associated with inhibition of NF-κB signaling, subsequent JNK activation and cell death (Nakano, Oshima et al. 1996, VanArsdale, VanArsdale et al. 1997, Hauer, Puschner et al. 2005, Kim, Nedospasov et al. 2005, Bista, Zeng et al. 2010).

While TNF receptors mediate gene expression only via the canonical NF-κB signaling pathway, LTβR signaling is able to induce gene expression via both the canonical and the alternative NF-κB pathway (Dejardin, Droin et al. 2002). The NF-κB family of transcription factors consists of 5 members that are active as heterodimers. On the one hand, LTβR signaling via the canonical NF-κB pathway occurs via context-dependent recruitment of specific TRAF proteins. These stimuli activate the IκB kinase (IKK) complex, consisting of IKKα,β and IKKγ/NEMO subunits, which phosphorylate and degrade the inhibitory molecule IκB that activates the dimeric transcription factor p50-RelA. On the other hand, LTβR mediates signaling via the alternative NF-κB pathway by interrupting constitutive NF-κB-inducing kinase (NIK) degradation. NIK activity leads to IKKα activation and subsequent conversion of the inactive dimeric p100-RelB into the active p52-RelB dimer (Dejardin, Droin et al. 2002, Hehlgans and Pfeffer 2005, Fernandes, Dejardin et al. 2016, Mitchell, Vargas et al. 2016).

The LTβR is expressed on non-lymphoid cells such as fibroblasts, epithelial cells, follicular DCs (fDCs), hepatocytes and cells of the myeloid lineage (monocytes, DCs and mast cells) but is absent on lymphocytes (Force, Walter et al. 1995, Ehlers, Holscher et al. 2003). However, the LTβR specific ligand $LT\alpha_1\beta_2$ is expressed on lymphocytes such as B-, T- and NK cells (Crowe, VanArsdale et al. 1994, Browning, Sizing et al. 1997, Ansel, Ngo et al. 2000). Signaling via the LTβR is critically involved in a variety of biological functions, ranging from organogenesis of peripheral lymphoid tissues and maintenance of secondary peripheral organ structure, to inflammation and tissue homeostasis to regulation of innate and adaptive immune responses against a variety of pathogens (Futterer, Mink et al. 1998, Ehlers, Holscher et al. 2003, Spahn, Maaser et al. 2004, Hehlgans and Pfeffer 2005, Sedy, Bekiaris et al. 2014,

Fernandes, Dejardin et al. 2016). In addition, an important role of LTβR signaling in liver regeneration and liver tumorigenesis was extensively studied during the last 15 years (Anders, Subudhi et al. 2005, Scarzello, Jiang et al. 2016, Sorg, Behnke et al. 2016).

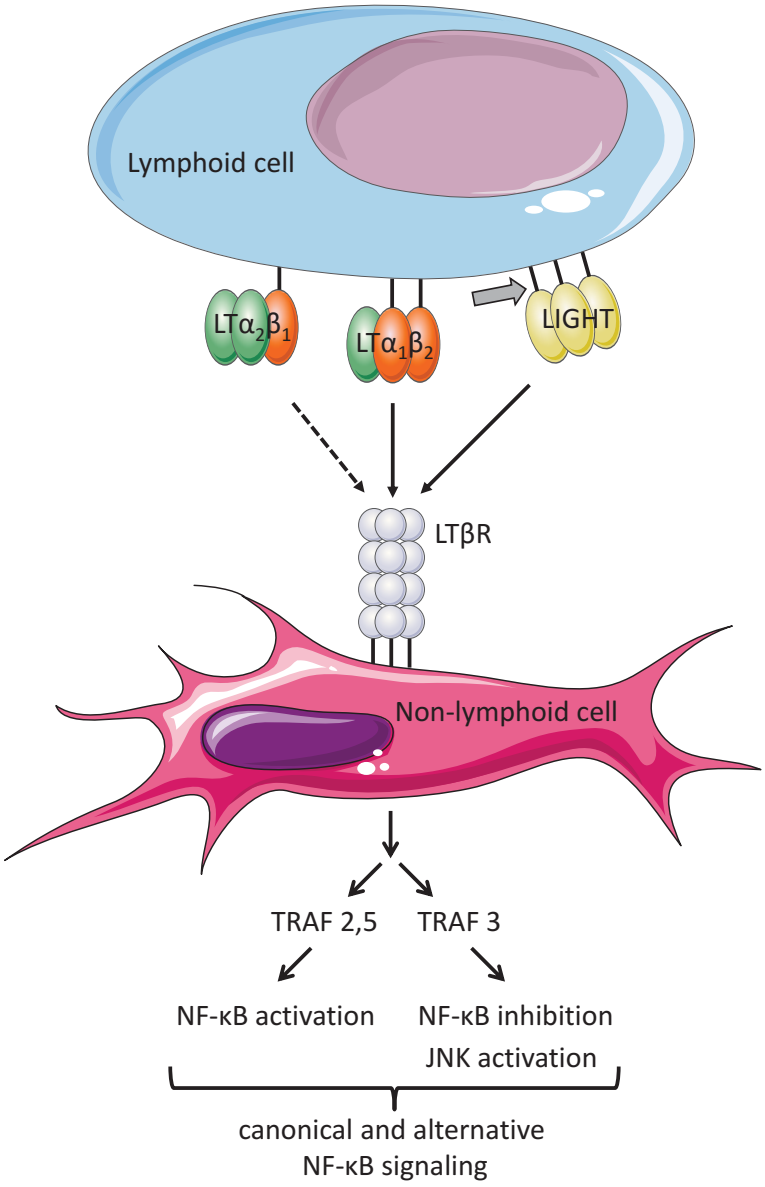


Figure 7: LTβR mediated signaling. The diagram illustrates cross-utilization of the LTβR, expressed on non-lymphoid cells, and its trimeric ligands $LT\alpha_2\beta_1$, $LT\alpha_1\beta_2$, and LIGHT that are expressed on lymphoid cells. Arrows define high affinity binding; dotted lines indicate low affinity binding and arrowheads define signaling directionality. Grey arrows indicate cleavage sites. While $LT\alpha_1\beta_2$ is an LTβR-specific ligand, the receptor also binds LIGHT and $LT\alpha_2\beta_1$, the latter with low affinity. Signal transduction occurs via TRAF adaptor protein recruitment with slightly different outcomes depending on the TRAF proteins involved. Signaling via TRAF2 and 5 results in activation of NF-κB signaling, whereas TRAF3 utilization results in inhibition of NF-κB and subsequent activation of JNK signaling. NF-κB signaling can occur via both the canonical and the alternative pathway with distinct downstream outcomes. The graphic was designed with Servier Medical Art License (http://creativecommons.org/licenses/by/3.0/). Changes were made to the provided elements.

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

Analysis of gene deficient animals, for instance LTβR deficient (LTβR$^{-/-}$) mice, is an important tool for the functional characterization of single gene products. Furthermore, gene deficient mice are valuable models for developing new and successful therapeutic approaches *in vivo*.

LTβR$^{-/-}$ mice lack lymph nodes, Peyer's patches and gut-associated lymphatic tissue (De Togni, Goellner et al. 1994, Alimzhanov, Kuprash et al. 1997, Koni, Sacca et al. 1997, Futterer, Mink et al. 1998, Tumanov, Kuprash et al. 2003, Kabashima, Banks et al. 2005). In addition, these mice show an altered architecture of the splenic microenvironment (Cheng, Onder et al. 2019). They have enlarged spleens, lack splenic marginal zones, T and B cell segregation and fDC networks (Futterer, Mink et al. 1998, Endres, Alimzhanov et al. 1999). B cells are no longer organized in follicles but intermixed with T cells. Moreover, LTβR$^{-/-}$ mice exhibit lymphocyte infiltrates in many organs, increased numbers of lymphocytes in the peritoneal cavity and peripheral blood, the latter reflected by increased B and T cell numbers. In contrast, they have reduced NK and DC cell numbers as well as impaired immunoglobulin (Ig) affinity maturation (Futterer, Mink et al. 1998, Wu, Sun et al. 2001). Analysis of LTβR$^{-/-}$ mice further revealed dysregulated bile acid homeostasis and a reduced capacity for liver regeneration following 70% hepatectomy (Anders, Subudhi et al. 2005, Sorg, Behnke et al. 2016). All these characteristics underline the importance of LTβR signaling in a variety of biological functions.

In particular, the LTβR is known for its important role in initiation of immune responses against various pathogens. LTβR$^{-/-}$ mice show pronounced defects in the defence against many pathogens (see below).

1.3.4 Role of the LTβR in infections

The important role of the LTβR in efficient initiation of host responses to a variety of pathogens has been demonstrated, encompassing bacteria such as *Listeria monocytogenes* (Ehlers, Holscher et al. 2003, Kursar, Janner et al. 2008, Kutsch, Degrandi et al. 2008) and *Mycobacterium tuberculosis* (Ehlers, Holscher et al. 2003), viruses such as *cytomegalovirus* (Banks, Rickert et al. 2006), Zika virus (Jin, Guo et al. 2018), and intracellular parasites such as *Plasmodium chabaudi* and *P. berghe*i (Krucken, Braun et al. 2005, Randall and Engwerda 2010) and *Leishmania donovani* and *L. major* (Xu, Liu et al. 2007, Stanley, de Labastida Rivera et al. 2011), and, last but not least *T. gondii* (Behnke, Sorg et al. 2017).

It has also been shown that other core members of the TNF/TNFR superfamily, namely LTα, TNF, and TNFRI, but not TNFRII are vital for host defence against the intracellular parasite *T. gondii* (Deckert-Schluter, Bluethmann et al. 1998, Schluter, Kwok et al. 2003, Hunter and

Sibley 2012). TNF and LTα are crucial for controlling *T. gondii* in the central nervous system, since mice deficient for LTα and TNF failed to control intracerebral parasites and succumbed to acute necrotizing toxoplasma encephalitis (Schluter, Kwok et al. 2003).

Behnke et al. show that LTβR mediated signaling is crucial for the overall survival in *T. gondii* infection: Lung and liver tissue of *T. gondii* infected LTβR$^{-/-}$ mice exhibit a marked exacerbation of organ pathology and increased numbers of cysts (Behnke, Sorg et al. 2017). Furthermore, a dysregulated expression profile of several cytokines known to be involved in activation of innate immune responses was observed in the serum of *T. gondii* infected LTβR$^{-/-}$ mice. The authors claim that the inability to induce appropriate early IFNγ responses as well as an inadequate upregulation of mGBPs causes the high mortality of *T. gondii* infected LTβR$^{-/-}$ mice (Behnke, Sorg et al. 2017).

However, the pathophysiology responsible for the increased susceptibility of LTβR$^{-/-}$ mice in *T. gondii* infection remains elusive. Further investigations are required to identify the molecular mechanisms and to aid in developing much needed new treatment and prevention options for human toxoplasmosis.

1.4 Clinical relevance of LTβR signaling

The LTβR network plays an important role in autoimmune diseases as multiple studies demonstrated its role in the pathogenesis of experimental autoimmune diseases (Gommerman and Browning 2003). Pharmacological inhibition of the LT pathway is shown to effectively reduce clinical severity in murine models of autoimmune diseases including experimental autoimmune encephalomyelitis (EAE) (Gommerman, Giza et al. 2003, Columba-Cabezas, Griguoli et al. 2006), collagen-induced arthritis (CIA) (Fava, Notidis et al. 2003), type I diabetes (Ettinger, Munson et al. 2001, Wu, Salomon et al. 2001), Uveitis (Shao, Fu et al. 2003), Sjögren's syndrome (Gatumu, Skarstein et al. 2009), colitis (Mackay, Browning et al. 1998) and graft vs. host disease (Tamada, Tamura et al. 2002).

Various publications (Benedict, Banks et al. 2001, Banks, Rickert et al. 2005, Kutsch, Degrandi et al. 2008, Schneider, Loewendorf et al. 2008, Li, Fu et al. 2015) highlight the interactions between the LTβR and type I IFN pathways, and point towards a pathogenic role of these pathways in the corresponding human diseases. Since a number of these diseases are associated with continuous type I IFN production which may be modulated by LTβ mediated signaling it will be important to further analyse inhibitors of the LT pathway as potential therapeutic agents in future treatment strategies (Gommerman, Browning et al. 2014). It is well known that IFNγ is crucial in host protection against pathogens and that the LTβR is important

for efficient initiation of host responses to a variety of pathogens, suggesting that an interaction between the LTβR and IFNγ, similar to LTβR and type I IFNs, might lead to new treatment options for different diseases as well as infections like toxoplasmosis. However, an interaction between the LTβR and the type II IFN pathways has not been described so far.

Toxoplasmosis is one of the most common infectious diseases, more widespread than malaria or tuberculosis, which is a serious global health problem (Flegr, Prandota et al. 2014). It affects about one third of the entire human population and is a severe opportunistic infection in immunocompromised patients. With the increasing number of immune-compromised patients, reactivation of latent tissue cysts can be observed more frequently. This is particularly life-threatening in organ transplant patients, as mortality ranges from 38% - 67% despite treatment (Gajurel, Dhakal et al. 2015). Over 50 different health disorders and diseases have been studied in the context of chronic toxoplasmosis e.g. schizophrenia (Torrey, Bartko et al. 2007, Zhu 2009, Hinze-Selch, Daubener et al. 2010, Tedla, Shibre et al. 2011, Fuglewicz, Piotrowski et al. 2017), Alzheimer's disease (Rao, Sridhar et al. 2007, Kusbeci, Miman et al. 2011) and central diabetes (Yamakawa, Yamashita et al. 1996, Oygur, Yilmaz et al. 1998, Nitta, Suzumura et al. 2006). To date there is no vaccine available to prevent or a drug to cure human toxoplasmosis. Anti-parasitic drugs for the treatment of toxoplasmosis are available, but taken alone these are not powerful enough and have to be taken in combination with other drugs. Dramatically, these supplemental drugs often have severe side effects such as myelotoxicity that require discontinuation of the therapy. Most importantly, so far no drug is available to eliminate quiescent tissue cysts (Djurkovic-Djakovic, Dupouy-Camet et al. 2019, Konstantinovic, Guegan et al. 2019).

It is therefore desirable to identify potent novel therapeutical candidates that would ideally be well-tolerated by pregnant women and newborns and could act on both tachyzoites and tissue cysts (Konstantinovic, Guegan et al. 2019). Understanding the molecular mechanisms of LTβR signaling could lead to a new understanding of the pathophysiology of toxoplasmosis and aid in developing sorely needed new treatment and prevention options such as vaccination strategies for human toxoplasmosis.

2. Aim of the thesis

The LTβR is known to be critically involved in various immunological processes, encompassing organogenesis and maintenance of secondary lymphoid organs, autoimmune diseases and immune responses against diverse pathogens (Futterer, Mink et al. 1998, Hehlgans and Pfeffer 2005, Gommerman, Browning et al. 2014, Fernandes, Dejardin et al. 2016, Behnke, Sorg et al. 2017). LTβR signaling has been shown to be crucially involved in efficient immune responses against *T. gondii*. Infected LTβR$^{-/-}$ mice exhibited virtually absent up-regulation of mGBPs, marked exacerbation of organ pathology, increased parasite load and deregulated cytokine expression profiles. This, and the inability to induce appropriate IFNγ responses such as adequate upregulation of IFNγ induced effector molecules that mediate essential functions in host cell defence (e.g. mGBPs) demonstrate that the LTβR is essential for the overall survival in *T. gondii* infection (Behnke, Sorg et al. 2017). However, a direct interaction between LTβR mediated and IFNγ signaling has not been identified so far.

Accordingly, the aim of this thesis was to further characterize and to survey innate and adaptive immune responses of LTβR$^{-/-}$ mice during *T. gondii* infection in detail. Therefore, *T. gondii* infection experiments of wildtype and LTβR$^{-/-}$ mice were performed in which cytokine expression and expression of IFNγ-induced effector molecules were extensively studied in different tissues. Since little is known about splenic immune cell populations in infected LTβR$^{-/-}$ mice, a comprehensive analysis of various cell populations as well as T cell functionality was assessed. Parasite specific antibody responses were investigated. These results subsequently led to the establishment of a passive immunization model. Finally, transcriptomics were performed to unravel novel, so far unknown links between LTβR mediated and IFNγ pathways during *T. gondii* infection and to gain further insights in LTβR orchestrated immune programs.

3. Publications

3.1 Lymphotoxin β receptor: A crucial role in innate and adaptive immune responses against *Toxoplasma gondii*
(manuscript submitted)

3.2 Lymphotoxin β receptor deficiency leads to dysregulation of anti-parasitic effector functions in *Toxoplasma gondii* infection
(manuscript to be submitted)

3.3 Indoleamine 2,3-Dioxygenase Activity During Acute Toxoplasmosis and the Suppressed T cell Proliferation in Mice
(Frontiers in Cellular and Infection Microbiology, 2019, 9:184)

In addition, the following publication has arisen from the present thesis.

Origin and differentiation trajectories of fibroblastic reticular cells in the splenic white pulp. Cheng HW, Onder L, Novkovic M, Soneson C, Lütge M, Pikor N, Scandella E, Robinson MD, Miyazaki JI, **Tersteegen A**, Sorg U, Pfeffer K, Rülicke T, Hehlgans T, Ludewig B. "" Nat Commun. 2019 Apr 15;10(1):1739. doi: 10.1038/s41467-019-09728-3. PMID: 30988302; PMCID: PMC6465367.

3.1 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

manuscript submitted

Impact factor

-

Own contributions to this work

Overall: 80%

Conducted all of the following experiments:

T. gondii infection experiments, qRT-PCRs (mRNA expression and parasite load), cytokine analysis, flow cytometry, immunoblot analysis, detection of *T. gondii* specific antibodies, serum transfer experiments, sample preparation for RNAseq

Other major contributions:

Design of experiments, manuscript preparation, data analysis

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

Abstract

LTβR mediated signaling plays an essential role in the initiation of immune responses to intracellular pathogens. In mice, the LTβR is crucial for surviving acute toxoplasmosis, however, up to now a functional analysis is lacking. Here, we demonstrate that the LTβR is a key regulator required for the intricate balance of adaptive immune responses. *T. gondii* infected LTβR$^{-/-}$ mice show globally altered IFNγ regulation, reduced IFNγ-controlled host effector molecule expression, impaired T cell differentiation/functionality and an absent anti-parasite specific IgG response resulting in a complete loss of immune control of *T. gondii* parasites. Reconstitution of LTβR$^{-/-}$ mice with toxoplasma immune serum significantly prolongs the survival in *T. gondii* infection. Interestingly, analysis of RNAseq data revealed a conceivable influence of *T. gondii* infection on the B cell response and isotype switching showing an important novel role for LTβR signaling in B cell mediated immune responses to control *T. gondii*.

Introduction

The LTβR, one of the core members of the tumor necrosis factor (TNF)/TNF receptor (TNFR) superfamily, is known to play an essential role in the organogenesis of peripheral lymphoid tissues and the maintenance of secondary peripheral organ structures but its function in mediating innate immune responses to many pathogens is also well-documented[1, 2, 3]. On the one hand, LTβR deficient (LTβR$^{-/-}$) mice lack lymph nodes and Peyer's patches, and show reduced NK- and DC cell numbers as well as impaired immunoglobulin (Ig) affinity maturation[4, 5]. On the other hand, LTβR deficiency results in pronounced defects in the defence also against *Listeria monocytogenes*, *Mycobacterium tuberculosis*[1], cytomegalovirus[6], LCMV[7] and Zika virus[8]. Since one third of the human population is infected with the obligate intracellular protozoan *Toxoplasma gondii* (*T. gondii*), toxoplasmosis causes a global health and economic burden[9]. Acute toxoplasmosis is usually inapparent or presents with mild flu-like symptoms; however, *T. gondii* infection can lead to life-threatening infections in immunocompromised individuals[10, 11]. After immune suppression, reactivation of chronic toxoplasmosis is also a serious medical complication. Primary infection of pregnant women can lead to congenital toxoplasmosis or cause miscarriage/stillbirth[10]. The early innate immune response to *T. gondii* is triggered by recognition of *T. gondii* associated molecules (e.g. profilin) by leukocytes, initiating cytokine production that consequently induces IFNγ production. Various cell types including NK cells[12], T cells[13], ILCs[14] and macrophages[15] have been described to produce IFNγ, which is known to play a vital role in *T. gondii* infection[16, 17, 18]. Induction of cell-autonomous effector mechanisms[19, 20], such as depletion of tryptophan[21] and reactive nitrogen production[22] which suppress *T. gondii* replication, are important for restricting parasite growth.

In addition, murine Guanylate-Binding Proteins (mGBPs) are known to be essential for pathogen containment as shown for *T. gondii*[23, 24, 25], *Listeria monocytogenes*[23], *Mycobacterium bovis BCG*, *Chlamydia muridarum*[26], *Shigella flexneri*[27] and other intracellular pathogens. Previous studies have shown that core members of the TNF/TNFR superfamily such as TNF and its receptors, LTβ and its receptor LTβR play an important part in the immune response to *T. gondii*[28, 29]. While it has been demonstrated that signaling via the LTβR is essential for the up-regulation of mGBPs after *T. gondii* infection as well as for overall survival[28], 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 IFNγ responses, impaired expression of anti-parasite 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 underlining a pivotal role for the humoral immune response in *T. gondii* infection. Furthermore, a transcriptomic based host-pathogen prediction model suggests that *T. gondii* negatively regulates the B cell response. These results lead to a new understanding of the 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 increases parasite burden and dysregulates cytokine expression.

LTβR$^{-/-}$ mice were highly susceptible to *T. gondii* infection succumbing by day 14 compared to wildtype C57Bl/6 (WT) mice (Supplementary Fig. 1). Interestingly, uninfected LTβR$^{-/-}$ mice showed increased spleen weight compared to WT mice, but during the course of infection, spleen weight of WT but not LTβR$^{-/-}$ mice increased markedly (Supplementary Fig. 2b - c). This increase was not due to higher splenocyte numbers but to inflammatory tissue swelling (Supplementary Fig. 2a) since the cellularity of the spleens of LTβR$^{-/-}$ animals was higher through day 7 *post infection* (*p.i.)* (Supplementary Fig. 2c). These observations suggested that the enhanced mortality might be reflected in parasite numbers in *T. gondii* infected animals during acute infection. We found the parasite burden significantly increased in lung, spleen and muscle tissue of LTβR$^{-/-}$ mice on day 10 *p.i.* (Fig. 1a). The increased parasite burden in different tissues of LTβR$^{-/-}$ mice points towards a failure of these mice to control parasite proliferation in the acute stage of infection. Next, we asked which factors could be responsible for this increased parasite burden and analyzed cytokine expression patterns in sera of infected mice (Fig. 1b & c). On days 4 and 7, WT mice compared to LTβR$^{-/-}$ mice showed a

marked and significant increase in IFNγ levels which decreased again by day 10 *p.i.* whereas LTβR$^{-/-}$ animals showed delayed upregulation in IFNγ levels (day 10 *p.i.*). Compared to WT mice, LTβR$^{-/-}$ mice also showed significantly increased levels of IFNβ and MCP-1/CCL2 on day 10 *p.i.* (Fig. 1b). Interestingly, baseline levels of the pro-inflammatory cytokines IL-1β, IL-6 and IL-27 (Fig. 1c) were increased in uninfected LTβR$^{-/-}$ mice compared to WT mice, suggesting a different immune activation status of these animals. Similar to IFNγ, there was a delayed but subsequently overshooting increase of IL-10 in WT compared to LTβR$^{-/-}$ animals on day 10 *p.i.* IL-1α, IL1-β, IL-6, and IL-23 did not seem to increase in WT animals during infection, but significantly increased in LTβR$^{-/-}$ mice (IL-1α & IL-23: day 4 *p.i.*; IL-1β: day 7 and day 10 *p.i.*; IL-6: day 4 - 10 *p.i.*). IL-27 levels rose only slightly in WT animals during infection whereas LTβR$^{-/-}$ animals showed a significant increase on days 4 and 10 *p.i.* IL-12p70 and IL-17A levels did not increase post infection in either cohort. These results indicate a generally dysregulated/activated immune status in LTβR$^{-/-}$ animals, which seems to be exacerbated after infection.

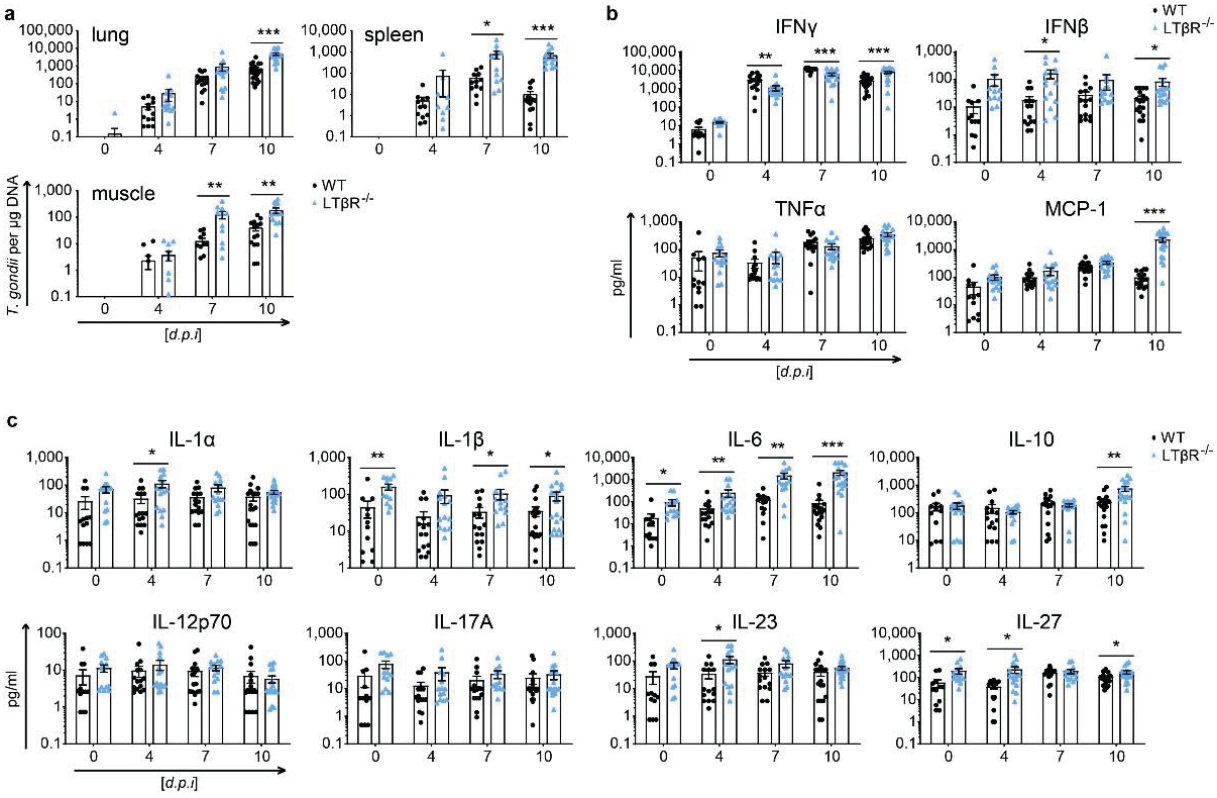


Fig. 1 | LTβR$^{-/-}$ mice show increased parasite load and dysregulated cytokine expression. a, qRT-PCR analysis of *T. gondii* DNA (assessing parasite load) in lung, spleen, muscle and brain tissue of uninfected (d0) and *T. gondii* infected (ME49, 40 cysts *i.p.*) WT and LTβR$^{-/-}$ mice (d0 - 7: n≥12, d10: n≥14). Expression of **b**, interferons, TNFα, and MCP-1 and **c**, interleukins in the serum of uninfected and *T. gondii* infected WT and LTβR$^{-/-}$ mice (d0 - 7: n≥12, d10: n=18) analyzed via qRT-PCR. Data shown represent four independent experiments; symbols represent individual animals, columns represent mean values and error bars represent ± SEM. Unpaired two-tailed Student's *t*-test was used for statistical analysis. *P<0.05, **P<0.01 and ***P<0.001.

LTβR deficiency leads to dysregulated expression of IFNγ-regulated effector molecules. The invasion of the lung during the acute phase of *T. gondii* infection (see Fig. 1a) led us to determine mRNA expression levels of cytokines, interleukins and IFNγ-regulated molecules. Baseline expression levels of IFNγ, LTα, LTβ and TNF were significantly increased in lungs of LTβR$^{-/-}$ animals (Fig. 2a), confirming the general dysregulated immune status described above. IFNγ levels were increased in both cohorts by day 4 *p.i.* although by day 10 decreased in WT yet sustained in LTβR$^{-/-}$ mice. IFN-β, LTα and -β mRNA expression did not increase in WT animals during infection, but were significantly elevated at day 10 *p.i.* in LTβR$^{-/-}$ animals.

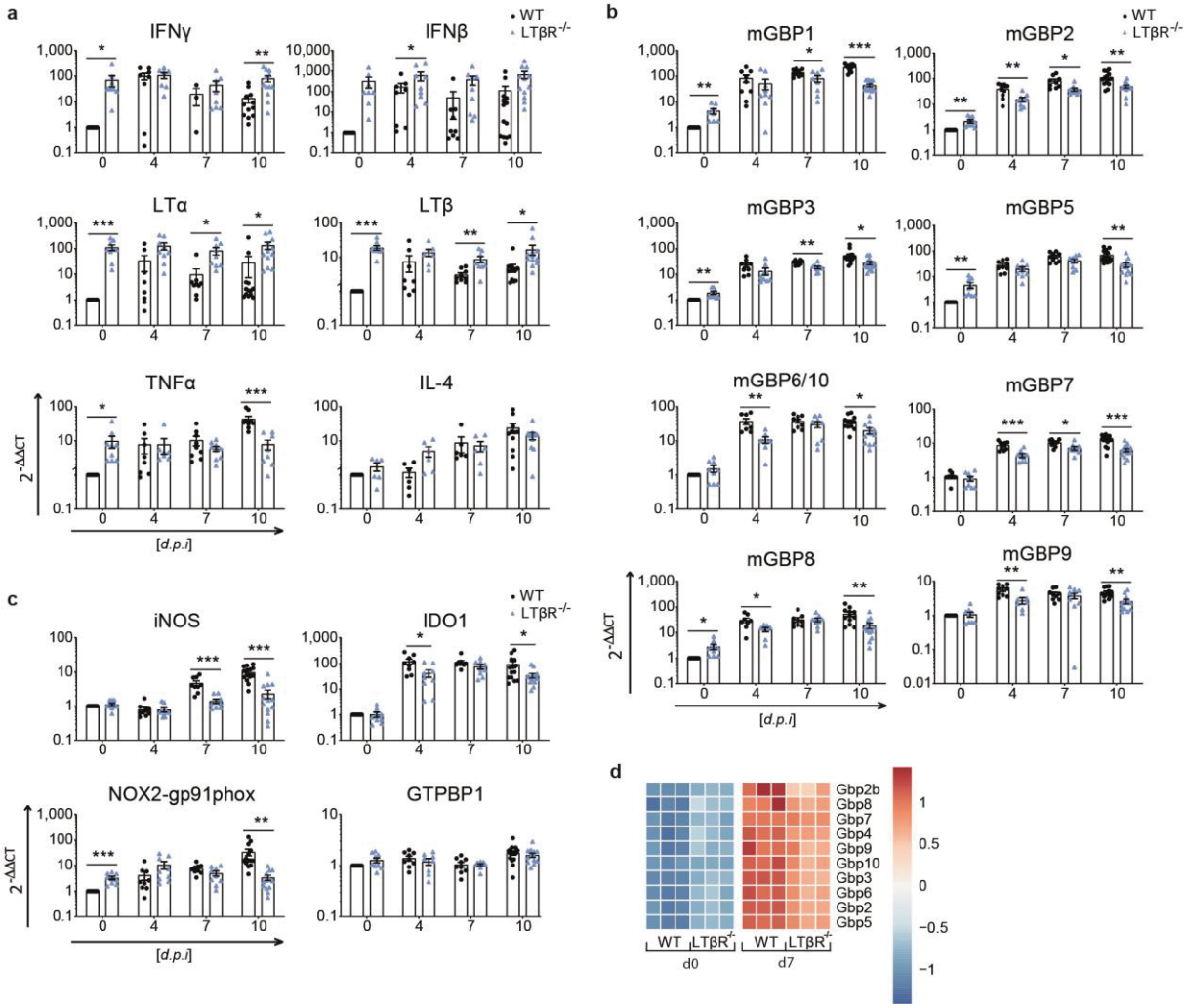


Fig. 2 | Decreased mRNA expression of IFNγ-mediated effector molecules in lungs of LTβR$^{-/-}$ mice. qRT-PCR analysis of **a**, interferons, lymphotoxins, TNFα and IL-4, **b**, murine Guanylate-Binding Proteins (mGBPs) and **c**, host effector molecules (iNOS, IDO1, NOX2-gp91phox) as well as an IFNγ unregulated GTPase (GTPBP1) 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, IL-12p40 n≥4 and IFNγ n≥3, d0 – 10 *p.i.*). Data shown represent four independent experiments; symbols represent individual animals, columns represent mean values and error bars represent ± SEM. Unpaired two-tailed Student's *t*-test was used for statistical analysis. *P<0.05, **P<0.01 and ***P<0.001. **d**, Heat map of differentially expressed mGBPs ("Wald" test & adjusted p-value of 0.1) based on RNAseq analysis of lung tissue from uninfected (d0) and *T. gondii* infected (d7 *p.i.*) WT and LTβR$^{-/-}$ mice (n=3).

Interestingly, uninfected LTβR$^{-/-}$ mice showed an increased baseline of TNF mRNA, that was not increased during the course of infection, in contrast to WT mice, IL-4 expression was not upregulated during infection in either cohort (Fig. 2a). IFNγ-regulated effector molecules are pivotal in *T. gondii* elimination[30], and have important immune response functions, i.e. modulating IFNγ-induced GTPases. In particular, the roles of mGBPs[23, 28] and effector molecules, such as IDO, iNOS and NOX2-gp91phox are well-documented[31, 32, 33]. Therefore, expression of IFNγ-regulated effector molecules was assessed by qRT-PCR. As expected, mRNA expression of all mGBPs analyzed increased during the course of infection, but LTβR$^{-/-}$ mice showed significantly reduced expression in all cases (Fig. 2b) for most time points. Again, baseline expression levels for some mGBPs (mGBP 1, 2, 3, 5, and 8) were slightly but significantly increased in LTβR$^{-/-}$ mice. Expression of the effector molecules iNOS, IDO1 and NOX2-gp91phox (Fig. 2c) was significantly lower in LTβR$^{-/-}$ mice on day 10 *p.i.* Interestingly, while increased baseline NOX2-gp91phox mRNA expression was detected in LTβR$^{-/-}$ mice, expression did not increase during *T. gondii* infection in contrast to WT animals which showed a significant increase (Fig. 2c). As a control, no significant change in expression of the IFNγ-independent GTPase GTPBP1 could be detected in WT or LTβR$^{-/-}$ mice during infection (Fig. 2c)[23]. RNAseq analysis of lung tissue further confirmed reduced mGBP RNA expression (Fig. 2d). In addition, *in vitro* experiments using WT and LTβR$^{-/-}$ mouse embryonic fibroblasts (MEFs) stimulated with IFNγ showed comparable mGBP expression in both cell types (Supplementary Fig. 3a) as well as similar recruitment of mGBP2 towards the *T. gondii* parasitophorous vacuole (PV) (Supplementary Fig. 3b), suggesting that in the presence of IFNγ mGBPs can function normally in LTβR$^{-/-}$ MEFs.

LTβR deficiency leads to dysregulation of IFNγ signaling. To further investigate altered gene expression, we analyzed lung tissue by RNAseq (Fig. 3a). Transcriptomic data confirmed differential expression of IFNγ regulated genes in *T. gondii* infected LTβR$^{-/-}$ mice (day 7 *p.i.*) compared to WT mice. Likewise, gene set enrichment analysis (GSEA) of RNAseq data shows an up-regulated response to interferons and interferon signaling in *T. gondii* infected (day 7 *p.i.*) WT mice (Supplementary Fig. 4). In order to investigate altered IFNγ signaling in LTβR$^{-/-}$ mice immunoblot assays were performed to analyze protein expression of prototype genes involved in IFNγR signaling as well as IFNγ-regulated genes in lung tissue (Fig. 3b & c). The level of STAT1, pSTAT1, pSTAT3, IRF-1, mGBP2, and mGBP7 increased in WT mice during the course of infection. In contrast, LTβR$^{-/-}$ animals showed a significant delay in the upregulation of these proteins. In WT animals, JAK1 and STAT3 expression increased until day 7 *p.i.* but decreased by day 10 *p.i.* In contrast, in LTβR$^{-/-}$ mice, expression of these proteins was higher in uninfected animals but did not increase early in infection. This confirms an altered IFNγ/IFNγR signaling axis occurs during infection. Surprisingly, the housekeeping genes β-actin and GAPDH showed increased protein expression during the course of *T. gondii*

infection. However, expression levels were comparable between WT and LTβR$^{-/-}$ mice on the individual days analyzed (Fig. 3b & c).

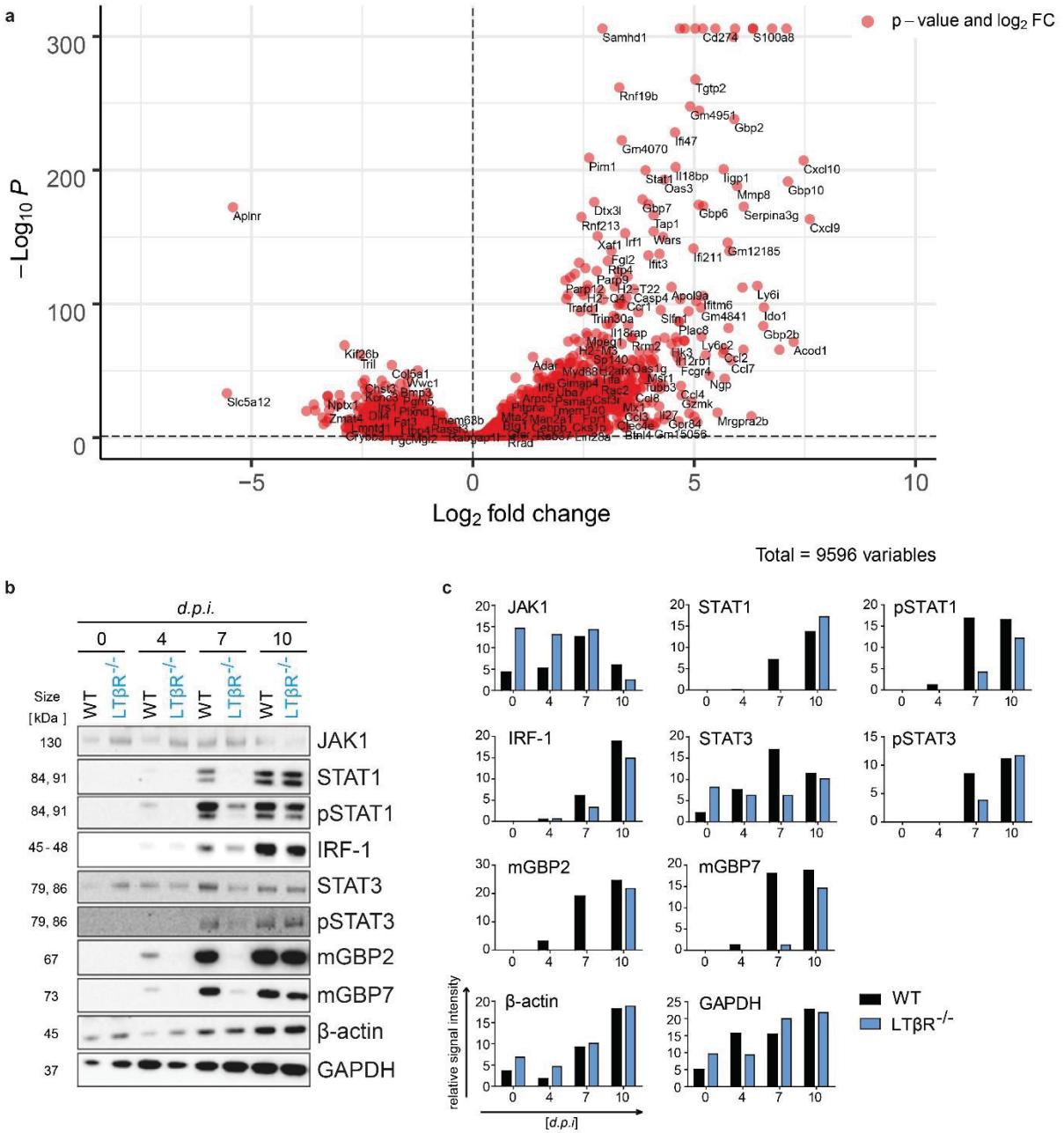
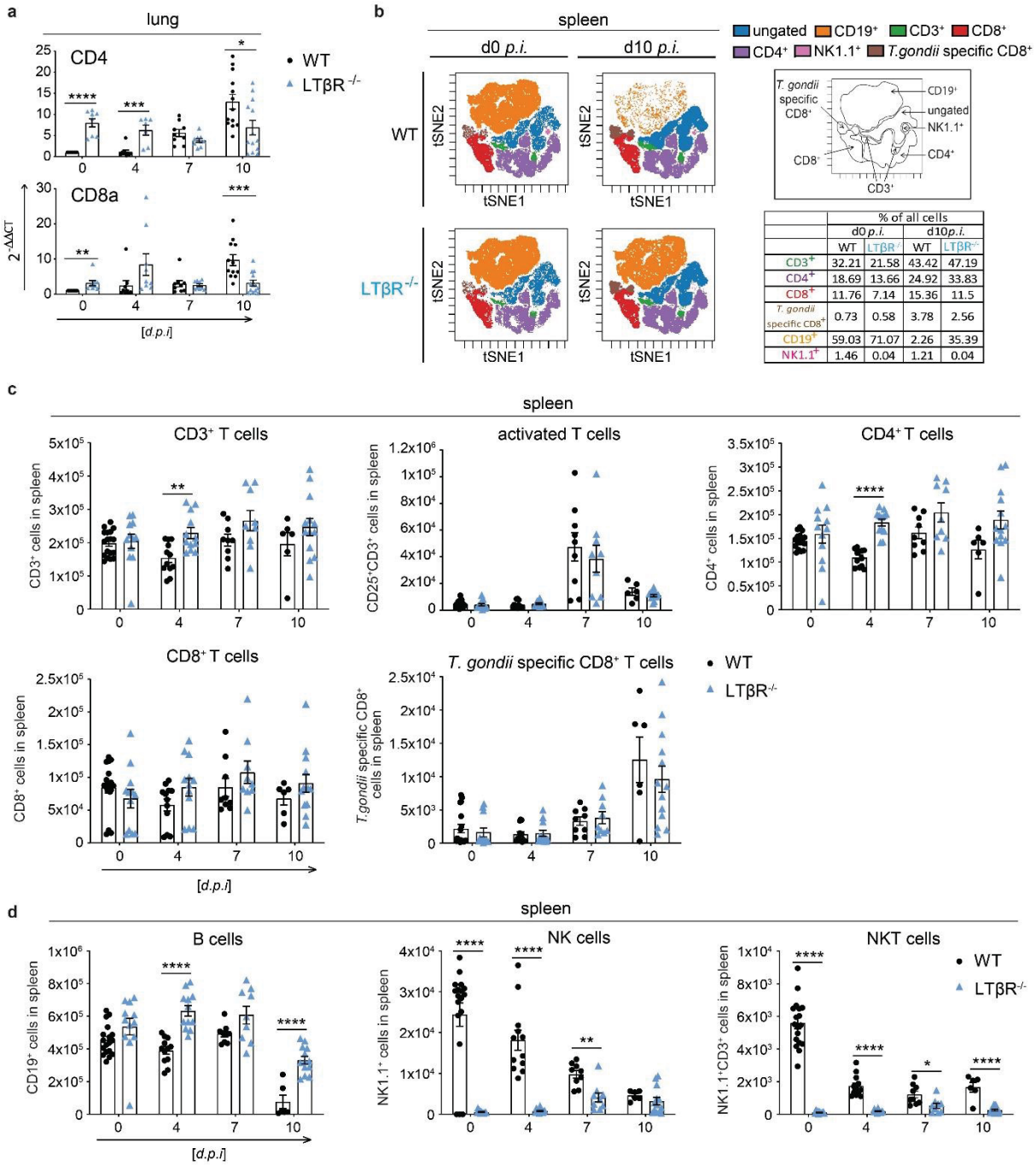


Fig. 3 | IFNγ response in lungs of infected WT and LTβR$^{-/-}$ mice. a, Volcano plot showing RNAseq data of lung tissue of infected WT mice correlated to infected LTβR$^{-/-}$ mice (d7 *p.i.;* n=3/group). Dashed horizontal black line represents an adjusted p-value of 0.1. **b**, Immunoblot analysis of proteins involved in or induced via the IFNγ signaling pathway in lung tissue from uninfected (d0) and *T. gondii* infected WT and LTβR$^{-/-}$ mice. **c**, Quantification of relative signal intensities of the immunoblot protein bands shown in **a** using ImageJ. Data shown in **a** & **b** are representative of three independent experiments.

LTβR deficiency does not influence the expansion of *T. gondii* specific T cells. qRT-PCR analysis also revealed significantly increased mRNA expression of T cell markers (CD4 and CD8a) in lung tissue of uninfected (day 0) LTβR$^{-/-}$ mice compared to WT mice as previously

reported[4]. In contrast, on day 10 *p.i.* WT mice showed increased amounts of CD4 and CD8a mRNA (Fig. 4a).



	% of all cells			
	d0 *p.i.*		d10 *p.i.*	
	WT	LTβR-/-	WT	LTβR-/-
CD3+	32.21	21.58	43.42	47.19
CD4+	18.69	13.66	24.92	33.83
CD8+	11.76	7.14	15.36	11.5
T. gondii specific CD8+	0.73	0.58	3.78	2.56
CD19+	59.03	71.07	2.26	35.39
NK1.1+	1.46	0.04	1.21	0.04

Fig. 4 | Dysregulated immune cell numbers in LTβR$^{-/-}$ mice. a, qRT-PCR analysis of CD4 and CD8a mRNA expression in lung tissue from uninfected (d0) and *T. gondii* infected (ME49, 40 cysts, *i.p.*) WT and LTβR$^{-/-}$ mice (d0 – 7 *p.i.*: n≥8, d10: n≥12). **b**, Representative tSNE plots from splenocytes of uninfected (d0) and *T. gondii* infected (d10 *p.i.*) WT and LTβR$^{-/-}$ mice. Clustered populations were identified using the indicated markers. **c**, Absolute cell numbers of CD3$^+$, activated, CD4$^+$, CD8$^+$, *T. gondii* specific CD8$^+$ T cells, and **d**, B- , NK-, and NKT cells in spleens of uninfected (d0) and *T. gondii* infected WT and LTβR$^{-/-}$ mice (d0 – 7 *p.i.*: n=12, d10 *p.i.*: n≥6) determined via flow cytometry. Data shown represent at least three independent experiments; symbols represent individual animals, columns represent mean values and error bars represent ± SEM. Unpaired two-tailed Student's *t*-test was used for statistical analysis. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Since splenomegaly is often observed in patients who die due to *T. gondii* infection[34] and also in infected WT mice (Supplementary Fig. 2a) we examined the different immune cell types in the spleen. Since total splenocyte numbers differed significantly between WT and LTβR$^{-/-}$ mice (Supplementary Fig. 2b) two different analysis approaches were performed. Splenocytes were measured using flow cytometry and analyzed either via an unbiased analysis of the data using tSNE (Fig. 4b) showing cell frequencies or manually via FlowJo (Fig. 4c & d) illustrating absolute cell numbers in the spleen. Unbiased analysis revealed a marked reduction of B cells (CD19^{+}) in WT spleens on day 10 *p.i.*, whereas no apparent change was observed in LTβR$^{-/-}$ mice between day 0 and day 10 (Fig. 4b, orange cluster). This was confirmed in a more detailed manual analysis, where LTβR$^{-/-}$ mice had significantly fewer reduced B cells on days 4 and 10 *p.i.* compared to WT mice (Fig. 4d). NK and NKT cells numbers were significantly higher in uninfected WT animals and declined during the course of infection as has been described previously[35] (Fig. 4c). As expected, both analyses confirmed the mentioned absence of NK and NKT cells in LTβR$^{-/-}$ mice[5, 36] (Fig. 4b & c). The low numbers of NK and NKT cell numbers did not change significantly in LTβR$^{-/-}$ mice during the infection. Since T cells are described to be essential to control *T. gondii* infection[37, 38], we analyzed different T cell populations (Fig. 4d). LTβR$^{-/-}$ mice showed comparable numbers for CD3^{+} and CD4^{+} T cells on days 0, 7 and 10 *p.i.* (Fig. 4d). Surprisingly, no differences between WT and LTβR$^{-/-}$ mice in absolute cell numbers of CD8^{+} T cells, activated T cells and especially *T. gondii* specific CD8^{+} T cells could be detected during *T. gondii* infection. This demonstrates that deficiency of the LTβR does not impact the expansion of T cells, especially parasite specific T cells during the acute stage of *T. gondii* infection.

Impaired T cell effector function in the absence of the LTβR. LTβR$^{-/-}$ mice are highly susceptible to *T. gondii* infection but possess comparable CD8^{+} and *T. gondii* specific CD8^{+} T cell numbers in the spleen (Fig. 4d). We determined whether T cells are fully functional with regard to their ability to produce IFNγ and cytotoxic granules and to degranulate upon stimulation. In order to address this question, splenocytes of infected mice (day 7 and 10 *p.i.*) remained unstimulated or were restimulated *ex vivo* with toxoplasma lysate antigen (TLA) before flow cytometry analysis (Fig. 5). After *ex vivo* TLA restimulation LTβR$^{-/-}$ mice compared to WT mice showed reduced numbers of CD4^{+} IFNγ producing T cells (day 7 and 10 *p.i.*), increased numbers of CD8^{+} IFNγ producing T cells (day 10 *p.i.*) and comparable numbers of *T. gondii* specific IFNγ producing T cells (Fig. 5a). While comparable CD8^{+} T cell numbers (Fig. 4d) and increased numbers of IFNγ producing CD8^{+} T cells (Fig. 5a) were detected in the spleen of LTβR$^{-/-}$ mice, restimulated splenocytes also showed reduced frequencies of granzyme B and perforin containing cytotoxic granules in CD8^{+} T cells (day 7 *p.i.*) as well as reduced frequencies of degranulating CD8^{+} T cells (day 10 *p.i.*; supplementary Fig. 5a). Also, after *ex vivo* TLA restimulation, slightly reduced frequencies of cytotoxic granule containing

IFNγ producing CD8⁺ T cells could be determined in LTβR$^{-/-}$ mice on day 7 *p.i.* (Supplementary Fig. 5b). Compared to WT mice, reduced frequencies of *T. gondii* specific T cells containing granzyme B or perforin (day 7 *p.i.*), but comparable frequencies on day 10 *p.i.* could be identified in spleens of LTβR$^{-/-}$ mice following *ex vivo* TLA restimulation (Fig. 5b). In addition, LTβR$^{-/-}$ mice showed significantly reduced frequencies of degranulating parasite specific T cells on day 10 *p.i.* (Fig. 5b). Together, these results demonstrate that in LTβR$^{-/-}$ mice T cell effector functionality is impaired after *T. gondii* infection in the CD4 well as CD8 T cell compartments.

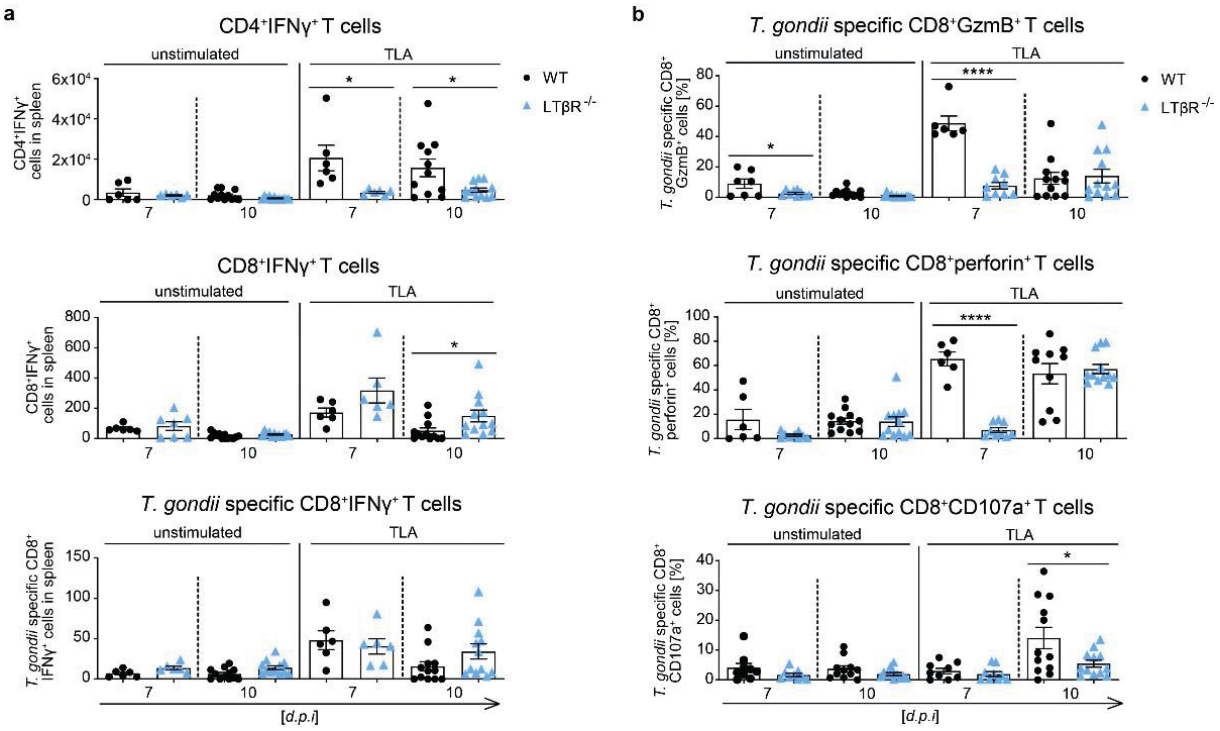
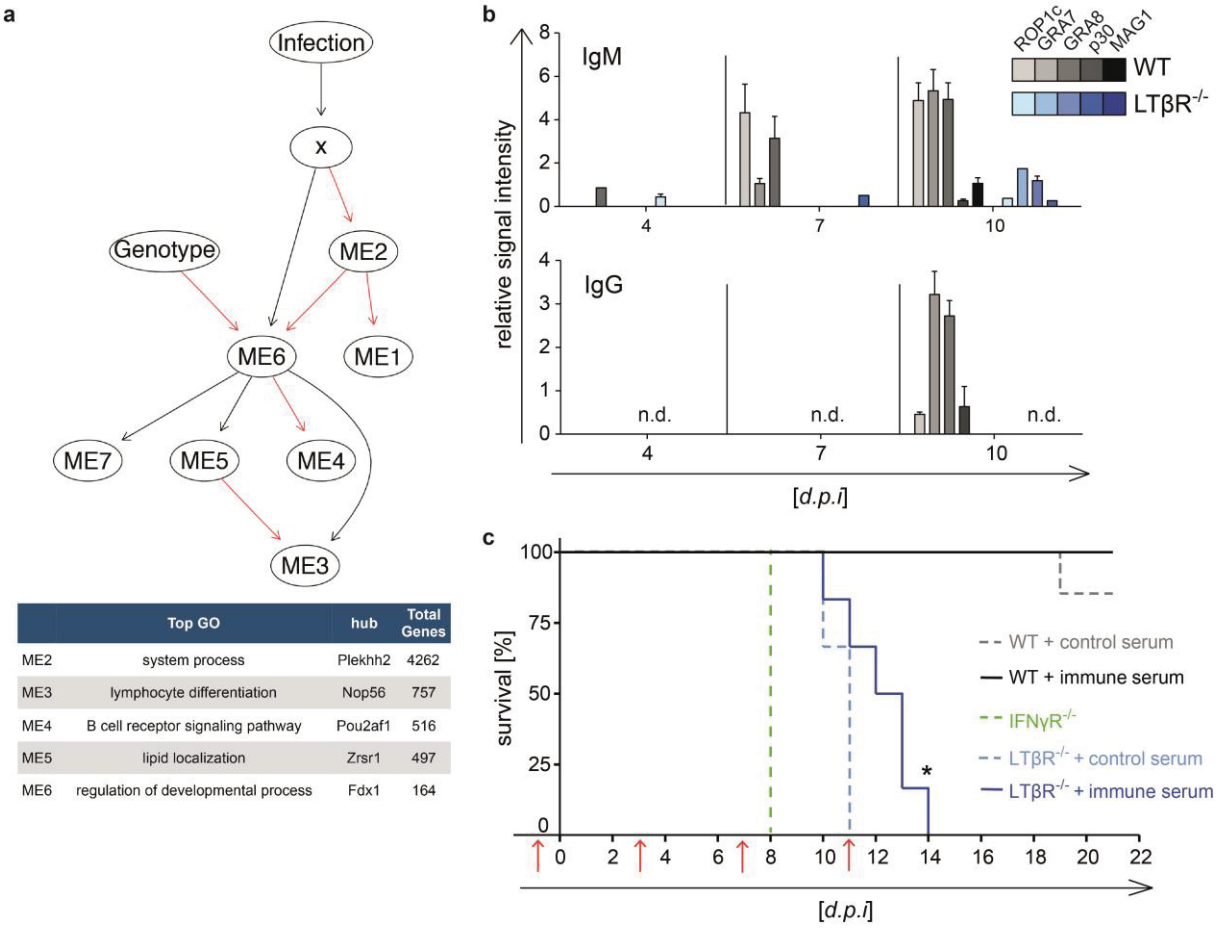


Fig. 5 | Reduced T cell functionality of LTβR$^{-/-}$ splenocytes. Intracellular staining of **a**, CD4⁺, CD8⁺ and *T. gondii* specific CD8⁺ IFNγ producing absolute T cells numbers and **b**, frequencies of cytotoxic granule (granzyme B or perforin) containing and degranulating (CD107a) *T. gondii* specific 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 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. Unpaired two-tailed Student's *t*-test was used for statistical analysis. *P<0.05 and ****P<0.0001.

LTβR deficiency abrogates *T. gondii* specific isotype class switching. RNAseq data of lung tissue from uninfected (day 0) and *T. gondii* infected (day 7 *p.i.*) WT and LTβR$^{-/-}$ animals was filtered for differentially expressed genes. Hierarchical clustering was performed, illustrated in supplementary Fig. 6a as sample dendrogram plus trait heat map for identification of possible outliers. All tested samples showed adequate clustering and could accordingly be clustered into uninfected and infected WT and LTβR$^{-/-}$ mice. Gene expression data was condensed into ten module eigengenes (ME0 - ME9; supplementary Fig. 6b) and used to

generate a host-pathogen network prediction model (Fig. 6a) displaying the relationship between modules (ME) and experimental conditions. This model captures the influence of *T. gondii* infection (Infection), LTβR genotype (Genotype), on host gene modules and total *T. gondii* genes (represented by "X") detected in each sample.



	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. 6 | Abrogated parasite specific isotype class switching and reconstitution of mice with *T. gondii* specific immune serum. a, Host-pathogen network prediction model generated on RNAseq 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 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≥20). Shown is a representative result of four independent experiments, bars represent mean values ± SEM. **c**, Transfer of serum (red arrows; d-1, 3, 7 and 11 *p.i.*) from uninfected donor WT mice (control serum) or from *T. gondii* infected 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. IFNγR$^{-/-}$ mice (n=3) served as infection controls. Data shown in **c** represent one experiment. **b** & **c**, Unpaired two-tailed Student's *t*-test was used for statistical analysis. *P<0.05 and n.d.= not detected.

Inspection of this model showed that LTβR being assigned to ME6 is suppressed by the LTβR$^{-/-}$ genotype, which fits with our experimental design. This model also predicts that high expression of ME6 genes in the WT mice, suppresses ME4 (Top GO “B cell receptor signaling pathway”), while enhancing ME3 (Top GO “lymphocyte differentiation”). This means that the loss of the LTβR slightly increases ME4 levels (supplementary Fig. 6c; Top GO “B cell receptor signaling pathway”) 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 (supplementary fig. 6d; Top GO “lymphocyte differentiation”) containing genes for “B cell activation” and “isotype switching” (Fig. 6a). Due to this highly surprising prediction, as well as increased B cell frequencies in the spleen on day 10 *p.i.* (Fig. 4c), 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. To analyze the role of antibodies as well as the capability of B cells for isotype switching in the immune response to *T. gondii* infection, the presence of immunoglobulin (Ig) M and IgG antibodies specific for *T. gondii* proteins was determined using line blots coated with specific recombinant *T. gondii* tachyzoite and bradyzoite antigens (ROP1c, GRA7, GRA8, p30 and MAG1). Surprisingly, LTβR$^{-/-}$ mice, as compared to WT mice, showed a delayed and reduced *T. gondii* specific IgM and an abrogated *T. gondii* specific IgG antibody response in serum during infection (day 4, 7, and 10 *p.i.;* Fig. 5b) that is in line with the prediction of the host-pathogen network. Since it has been described that a *T. gondii* specific IgG response is required for a reduction of the parasite burden[39], we treated LTβR$^{-/-}$ mice with immune serum (serum from *T. gondii* infected WT animals) and monitored the survival after *T. gondii* infection (Fig. 5c). In fact, our experiment showed that reconstituted LTβR$^{-/-}$ mice treated with immune serum exhibit prolonged survival (up to day 14 *p.i.*) compared to littermates that received control serum (from uninfected WT donor mice) which died by day 11 *p.i.* Furthermore, all reconstituted WT mice treated with immune serum (Fig. 5c) survived until day 21 *p.i.* compared to control animals (WT mice + control serum) where one animal succumbed to *T. gondii* infection by day 21, demonstrating the relevance of a *T. gondii* specific IgG antibody response. IFNγR$^{-/-}$ mice served as infection control and succumbed as expected around day 8 *p.i.*[40].

Discussion

The results obtained in this study have revealed a profoundly deficient immune response of LTβR$^{-/-}$ mice to *T. gondii*. This deficiency is characterized by a dysregulated IFNγ driven antimicrobial response, an impaired effector T cell response and a virtually abrogated immunoglobulin IgG class switching. Furthermore, serum transfer from infected WT donor mice showed a protective effect on survival. Importantly, *T. gondii* challenged LTβR$^{-/-}$ mice revealed an unsuspected role for the humoral immune response in protection from toxoplasmosis.

Our results support the idea that the LTβR pathway regulates the protective IFNγ response to *T. gondii*. In the absence of the LTβR-IFNγ response the parasite burden increases corresponding to a significant delay in IFNγ and altered gene upregulation detected in the lung after infection. By day 10 *p.i.* IFNγ levels in WT animals are markedly decreased, most probably reflecting control of parasite proliferation and transition to the chronic phase of infection. In contrast, IFNγ regulated expression of molecules important for cellular effector functions, such as iNOS, IDO1 and NOX2-gp91phox, as well as the upregulation of mGBPs, which are essential for an efficient immune response to *T. gondii*[24, 25, 41] are significantly reduced in LTβR$^{-/-}$ mice. Although LTβR$^{-/-}$ mice showed comparable IFNγ levels to WT mice in lung tissue during the early phase of infection, IFNγ signaling (STAT1/pSTAT1, IRF-1) and target gene expression (mGBP) were significantly delayed. The delayed IFNγ response in LTβR$^{-/-}$ mice is in line with the finding that LTβR$^{-/-}$ mice lack NK cells (Fig. 4)[5, 42]. We interpret our data in light of the NK cell deficiency that the delayed IFNγ response in LTβR$^{-/-}$ mice is caused in part by the absence of IFNγ producing NK cells, which are known to be an essential innate immune response to *T. gondii*[43]. Importantly, the reduced number of IFNγ producing CD4^{+} T cells and functional CD8^{+} cytotoxic lymphocytes (granzyme, perforin) strongly implies that cytotoxic T cell mediated killing is severely impaired in LTβR$^{-/-}$ animals. This further aggravates the LTβR insufficiency in host defense. Neither NK or T cells express the LTβR indicating the effect is indirect perhaps mediated by chemokines and adhesion proteins to attract and localize effector cells to the infected microenvironment. The equivalent response of infected WT and LTβR$^{-/-}$ MEFs to IFNγ argues that the LTβR deficiency does not impair signaling and downstream effector mechanisms. Thus the production of IFNγ is the critical point of the LTβR mechanism of action. Together these results suggest that the dysregulation of the early, IFNγ dependent immune response pathway is responsible for the increased susceptibility of LTβR$^{-/-}$ mice to *T. gondii* infection. These data establish the LTβR signaling mediated immune control of intracellular pathogens depends on IFNγ production. Our results indicate a role for the LTβR in regulating the production of protective antibodies to *T. gondii*. LTβR$^{-/-}$ mice lack lymph nodes and have disorganized architecture in the spleen[4].

The loss of LTβR expression and signaling in antigen-presenting dendritic cells and chemokine secretion by specialized stromal cells (follicular reticular cells) may hamper the ability to coordinate the germinal center reaction[44, 45] required for immunoglobulin class switch and affinity maturation[4]. Protective B cells appear to play a significant role in chronic rather than the acute *T. gondii* infection in some models[37, 38, 39, 46]. The host-pathogen network prediction model we generated from *T. gondii* infected mice indicates the loss of the LTβR inhibits B cell responses including isotype switching in *T. gondii* infection. We validated the predictive ability of the network model by demonstrating that T gondii infected LTβR$^{-/-}$ mice produced less IgM compared to WT mice, and no detectable specific IgG. Since IgG producing B cells migrate from the spleen into the bone marrow, it is conceivable that mature B cells cannot emigrate from the spleen into the bone marrow due to missing class switch, which would explain the less reduced B cell numbers in spleens of infected LTβR$^{-/-}$ mice compared to WT mice (Fig. 4b & d). LTβR$^{-/-}$ mice express reduced amounts of IFNγ which is known to stimulate the isotype class switch to IgG2a[47]. However, the absence of the ability to perform class switching may not (only) be due to reduced IFNγ production but also to the absence of the required microenvironment, since LTβR$^{-/-}$ mice lack germinal centers in the spleen. Indeed, the fact that the survival of LTβR$^{-/-}$ animals can be significantly prolonged by transfer of immune serum, containing *T. gondii* specific antibodies emphasizes the importance of a B cell-mediated immune response in the defense against *T. gondii.* Furthermore and unexpectedly, our host-pathogen prediction network indicates that *T. gondii* infection suppresses B cell responses in WT animals. This result points towards an unknown *T. gondii* strategy to evade the host immune system. *T. gondii* specific suppression of B cell responses, may allow dissemination and cyst formation the brain or muscle tissue to establish chronic infection[48].

To summarize, we show that the loss of LTβR signaling results in a depressed IFNγ response, impaired T cell functionality and the failure to induce parasite protective IgG antibodies leading to an increase in parasite burden and fatal *T. gondii* infection. Accordingly, we conclude that both intact T and B cell responses are essential for parasite clearance and survival. Thus, a deficiency in LTβR controlled pathways leads to a form of severe combined immune deficiency. Further understanding of this complex interplay between LTβR and IFNγ will provide new insights into the pathogenesis of *T. gondii* and provide novel therapeutics and vaccine strategies.

Methods

Mice. LTβR$^{-/-}$ mice were previously described[4] and are backcrossed for at least 10 generations onto a C57BL/6 background. Wild-type (WT) littermates were used as controls. Mice were kept under specific pathogen-free conditions (SPF) in the animal facility at the Heinrich Heine University Düsseldorf and were 8-16 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# 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 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 post infection (*d.p.i*), uninfected mice served as controls. After euthanasia (100 mg/kg Ketamin, 10 mg/kg Xylazin, 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. TgB1 primers and probe (Metabion) were used to amplify a defined section of the 35-fold repetitive B1 gene from *T. gondii* and are listed in Supplementary Table 1. The *T. gondii* standard curve was used to determine B1 amplification for calculation of parasite load.

Cytokine measurement. Cytokines MCP-1, IFNγ, IFNβ, IL-1α, IL-1β, IL-6, IL-10, IL12p70, IL-17A, IL-23, IL-27, and TNF were measured using the LEGENDplex™ Mouse Inflammation Panel (BioLegend®) according to the manufacturer's protocol.

Real-time qRT-PCR. Total RNA was isolated from tissues using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was reversely transcribed using M-MLV reverse transcriptase (Invitrogen). qRT-PCR was performed on the Bio-Rad CFX-96 Touch-Real-Time Detection System. Primer sequences and corresponding probes (Metabion, Roche & TipMolBIOL) are listed in Supplementary Table 1. Results are expressed relative to expression in untreated WT mice normalized to β-*actin* ($2^{-\Delta\Delta CT}$).

RNAseq analysis. Lung tissue of uninfected (d0) und *T. gondii* infected (ME49 strain, 40 cysts*, i.p.*) WT and LTβR$^{-/-}$ mice was obtained and RNA sequencing was performed on a HiSeq3000 device. Mouse and *T. gondii* transcripts were quantified from fastq files using Salmon with default settings and GCbias 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 by 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 datasets were used to generate the host-pathogen network as described[49]. Prior to network generation, the 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 this numeric data was merged to module eigengenes using the Data_Prep function of GmicR [Supplementary Figure 6]. 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 PBS containing cOmplete™ Protease Inhibitor Cocktail (Roche) using the Precellys® homogenizer (Bertin). 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 4-12% SDS-PAGE, followed by electrophoretic transfer to nitrocellulose membranes before blocking and incubation with primary antibodies listed in Supplementary Table 2. 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®[50] (Mountain View) was used for visualization of flow cytometry data. 60,000 events per sample were analyzed (parameters: iterations 2,400, perplexity 80, Theta 0.5) before overlaid dot plots were generated.

Flow cytometry. Single-cell suspended splenocytes 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 purchased from BD Bioscience), was performed. For intracellular staining splenocytes were incubated for 20 h with toxoplasma lysate antigen (TLA, 15 µg/ml) before adding brefeldin A (eBioscience™) for an additional 4 hours. 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-IFNγ (XMG1.2), anti-granzyme B (QA16A02), and anti-perforin (S16009A) (all purchased from BioLegend) using 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.

Detection of *T. gondii* specific antibodies. *Recom*Line *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. Serum from naïve donor mice (control serum) or WT mice infected with 20 cysts of the ME49 strain of *T. gondii* (immune serum) was collected. Acceptor WT and LTβR$^{-/-}$ mice were reconstituted intraperitoneally with 0.2 ml serum one 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 IFNγ$^{-/-}$ 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 (Version8, GraphPad) using two-tailed Student's *t*-test. Symbols represent individual animals, bars show mean values ± SEM. P values of ≤0.05 were considered statistically significant and marked with asterisks. P values of ≥0.05 were considered statistically not significant and were not specifically marked.

Life Science Reporting Summary. Further information on experimental design and reagents is available in the Life Sciences Reporting Summary.

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

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Author contributions

A.T. performed and analyzed all experiments, except for Fig. 2d, Fig. 3a, Fig.6a, supplementary Fig. 4 and Fig. 6; RVS developed the immune network models for analysis of RNAseq datasets. M.H. performed experiments illustrated in supplementary Fig. 3. P.P. and K.K. performed RNA sequencing. A.T., U.R.S. and K.P. wrote the manuscript with input from D.D. and C.F.W; K.P., U.R.S. and A.T. designed the study.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper.

References

1. Ehlers S, Holscher C, Scheu S, Tertilt C, Hehlgans T, Suwinski J, et al. The lymphotoxin beta receptor is critically involved in controlling infections with the intracellular pathogens Mycobacterium tuberculosis and Listeria monocytogenes. *J Immunol* 2003, **170**(10): 5210-5218.

2. Spahn TW, Maaser C, Eckmann L, Heidemann J, Lugering A, Newberry R, et al. The lymphotoxin-beta receptor is critical for control of murine Citrobacter rodentium-induced colitis. *Gastroenterology* 2004, **127**(5): 1463-1473.

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

4. Futterer A, Mink K, Luz A, Kosco-Vilbois MH, Pfeffer K. The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity* 1998, **9**(1): 59-70.

5. Wu Q, Sun Y, Wang J, Lin X, Wang Y, Pegg LE, et al. Signal via lymphotoxin-beta R on bone marrow stromal cells is required for an early checkpoint of NK cell development. *J Immunol* 2001, **166**(3): 1684-1689.

6. Banks TA, Rickert S, Ware CF. Restoring immune defenses via lymphotoxin signaling: lessons from cytomegalovirus. *Immunol Res* 2006, **34**(3): 243-254.

7. Puglielli MT, Browning JL, Brewer AW, Schreiber RD, Shieh WJ, Altman JD, et al. Reversal of virus-induced systemic shock and respiratory failure by blockade of the lymphotoxin pathway. *Nature medicine* 1999, **5**(12): 1370-1374.

8. Jin L, Guo X, Shen C, Hao X, Sun P, Li P, et al. Salivary factor LTRIN from Aedes aegypti facilitates the transmission of Zika virus by interfering with the lymphotoxin-beta receptor. *Nat Immunol* 2018, **19**(4): 342-353.

9. Flegr J, Prandota J, Sovickova M, Israili ZH. Toxoplasmosis--a global threat. Correlation of latent toxoplasmosis with specific disease burden in a set of 88 countries. *PLoS One* 2014, **9**(3): e90203.

10. Montoya JG, Liesenfeld O. Toxoplasmosis. *Lancet* 2004, **363**(9425): 1965-1976.

11. Sasai M, Yamamoto M. Pathogen recognition receptors: ligands and signaling pathways by Toll-like receptors. *International reviews of immunology* 2013, **32**(2): 116-133.

12. Gaddi PJ, Yap GS. Cytokine regulation of immunopathology in toxoplasmosis. *Immunol Cell Biol* 2007, **85**(2): 155-159.

13. Denkers EY. T lymphocyte-dependent effector mechanisms of immunity to Toxoplasma gondii. *Microbes Infect* 1999, **1**(9): 699-708.

14. Ivanova DL, Denton SL, Fettel KD, Sondgeroth KS, Munoz Gutierrez J, Bangoura B, et al. Innate Lymphoid Cells in Protection, Pathology, and Adaptive Immunity During Apicomplexan Infection. *Front Immunol* 2019, **10**: 196.

15. Darwich L, Coma G, Pena R, Bellido R, Blanco EJ, Este JA, et al. Secretion of interferon-gamma by human macrophages demonstrated at the single-cell level after costimulation with interleukin (IL)-12 plus IL-18. *Immunology* 2009, **126**(3): 386-393.

16. Suzuki Y, Orellana MA, Schreiber RD, Remington JS. Interferon-gamma: the major mediator of resistance against Toxoplasma gondii. *Science* 1988, **240**(4851): 516-518.

17. Yap GS, Sher A. Cell-mediated immunity to Toxoplasma gondii: initiation, regulation and effector function. *Immunobiology* 1999, **201**(2): 240-247.

18. Saeij JP, Frickel EM. Exposing Toxoplasma gondii hiding inside the vacuole: a role for GBPs, autophagy and host cell death. *Curr Opin Microbiol* 2017, **40**: 72-80.

19. Daubener W, Remscheid C, Nockemann S, Pilz K, Seghrouchni S, Mackenzie C, et al. Anti-parasitic effector mechanisms in human brain tumor cells: role of interferon-gamma and tumor necrosis factor-alpha. *Eur J Immunol* 1996, **26**(2): 487-492.

20. Fox BA, Gigley JP, Bzik DJ. Toxoplasma gondii lacks the enzymes required for de novo arginine biosynthesis and arginine starvation triggers cyst formation. *Int J Parasitol 2004*, **34**(3): 323-331.

21. Pfefferkorn ER, Rebhun S, Eckel M. Characterization of an indoleamine 2,3-dioxygenase induced by gamma-interferon in cultured human fibroblasts. *J Interferon Res* 1986, **6**(3): 267-279.

22. Scharton-Kersten TM, Yap G, Magram J, Sher A. Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen Toxoplasma gondii. *J Exp Med* 1997, **185**(7): 1261-1273.

23. Degrandi D, Konermann C, Beuter-Gunia C, Kresse A, Wurthner J, Kurig S, et al. Extensive characterization of IFN-induced GTPases mGBP1 to mGBP10 involved in host defense. *J Immunol* 2007, **179**(11): 7729-7740.

24. Steffens N, Beuter-Gunia C, Kravets E, Reich A, Legewie L, Pfeffer K, et al. Essential Role of mGBP7 for Survival of Toxoplasma gondii Infection. *mBio* 2020, **11**(1).

25. Yamamoto M, Okuyama M, Ma JS, Kimura T, Kamiyama N, Saiga H, et al. A cluster of interferon-gamma-inducible p65 GTPases plays a critical role in host defense against Toxoplasma gondii. *Immunity* 2012, **37**(2): 302-313.

26. Finethy R, Jorgensen I, Haldar AK, de Zoete MR, Strowig T, Flavell RA, et al. Guanylate binding proteins enable rapid activation of canonical and noncanonical inflammasomes in Chlamydia-infected macrophages. *Infect Immun* 2015, **83**(12): 4740-4749.

27. Wandel MP, Pathe C, Werner EI, Ellison CJ, Boyle KB, von der Malsburg A, et al. GBPs Inhibit Motility of Shigella flexneri but Are Targeted for Degradation by the Bacterial Ubiquitin Ligase IpaH9.8. *Cell Host Microbe* 2017, **22**(4): 507-518 e505.

28. Behnke K, Sorg UR, Gabbert HE, Pfeffer K. The Lymphotoxin beta Receptor Is Essential for Upregulation of IFN-Induced Guanylate-Binding Proteins and Survival after Toxoplasma gondii Infection. *Mediators Inflamm* 2017, **2017**: 7375818.

29. Schluter D, Kwok LY, Lutjen S, Soltek S, Hoffmann S, Korner H, et al. Both lymphotoxin-alpha and TNF are crucial for control of Toxoplasma gondii in the central nervous system. *J Immunol* 2003, **170**(12): 6172-6182.

30. Yarovinsky F. Innate immunity to Toxoplasma gondii infection. *Nat Rev Immunol* 2014, **14**(2): 109-121.

31. Ratna A, Arora SK. Leishmania recombinant antigen modulates macrophage effector function facilitating early clearance of intracellular parasites. *Trans R Soc Trop Med Hyg* 2016, **110**(10): 610-619.

32. Koo SJ, Chowdhury IH, Szczesny B, Wan X, Garg NJ. Macrophages Promote Oxidative Metabolism To Drive Nitric Oxide Generation in Response to Trypanosoma cruzi. In*fect Immun* 2016, **84**(12): 3527-3541.

33. Ufermann CM, Domrose A, Babel T, Tersteegen A, Cengiz SC, Eller SK, et al. Indoleamine 2,3-Dioxygenase Activity During Acute Toxoplasmosis and the Suppressed T Cell Proliferation in Mice. *Front Cell Infect Microbiol 2019*, **9**: 184.

34. Weiss LM, Dubey JP. Toxoplasmosis: A history of clinical observations. *Int J Parasitol* 2009, **39**(8): 895-901.

35. Park E, Patel S, Wang Q, Andhey P, Zaitsev K, Porter S, et al. Toxoplasma gondii infection drives conversion of NK cells into ILC1-like cells. *eLife* 2019, **8**.

36. Vallabhapurapu S, Powolny-Budnicka I, Riemann M, Schmid RM, Paxian S, Pfeffer K, et al. 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* 2008, **38**(12): 3508-3519.

37. Pittman KJ, Knoll LJ. Long-Term Relationships: the Complicated Interplay between the Host and the Developmental Stages of Toxoplasma gondii during Acute and Chronic Infections. *Microbiology and molecular biology reviews : MMBR* 2015, **79**(4): 387-401.

38. Sher A, Denkers EY, Gazzinelli RT. Induction and regulation of host cell-mediated immunity by Toxoplasma gondii. *Ciba Foundation symposium* 1995, **195**: 95-104; discussion 104-109.

39. Park J, DeLong JH, Knox JJ, Konradt C, Wojno EDT, Hunter CA. Impact of Interleukin-27p28 on T and B Cell Responses during Toxoplasmosis. *Infect Immun* 2019, **87**(12).

40. Deckert-Schluter M, Rang A, Weiner D, Huang S, Wiestler OD, Hof H, et al. Interferon-gamma receptor-deficiency renders mice highly susceptible to toxoplasmosis by decreased macrophage activation. *Lab Invest* 1996, **75**(6): 827-841.

41. Degrandi D, Kravets E, Konermann C, Beuter-Gunia C, Klumpers V, Lahme S, et al. Murine guanylate binding protein 2 (mGBP2) controls Toxoplasma gondii replication. *Proc Natl Acad Sci U S A* 2013, **110**(1): 294-299.

42. Iizuka K, Chaplin DD, Wang Y, Wu Q, Pegg LE, Yokoyama WM, et al. Requirement for membrane lymphotoxin in natural killer cell development. *Proc Natl Acad Sci U S A* 1999, **96**(11): 6336-6340.

43. Gigley JP. The Diverse Role of NK Cells in Immunity to Toxoplasma gondii Infection. *PLoS Pathog* 2016, **12**(2): e1005396.

44. Wang YG, Kim KD, Wang J, Yu P, Fu YX. Stimulating lymphotoxin beta receptor on the dendritic cells is critical for their homeostasis and expansion. *J Immunol* 2005, **175**(10): 6997-7002.

45. Endres R, Alimzhanov MB, Plitz T, Futterer A, Kosco-Vilbois MH, Nedospasov SA, et al. Mature follicular dendritic cell networks depend on expression of lymphotoxin beta receptor by radioresistant stromal cells and of lymphotoxin beta and tumor necrosis factor by B cells. *J Exp Med* 1999, **189**(1): 159-168.

46. Chen M, Mun HS, Piao LX, Aosai F, Norose K, Mohamed RM, et al. Induction of protective immunity by primed B-1 cells in Toxoplasma gondii -infected B cell-deficient mice. *Microbiol Immunol* 2003, **47**(12): 997-1003.

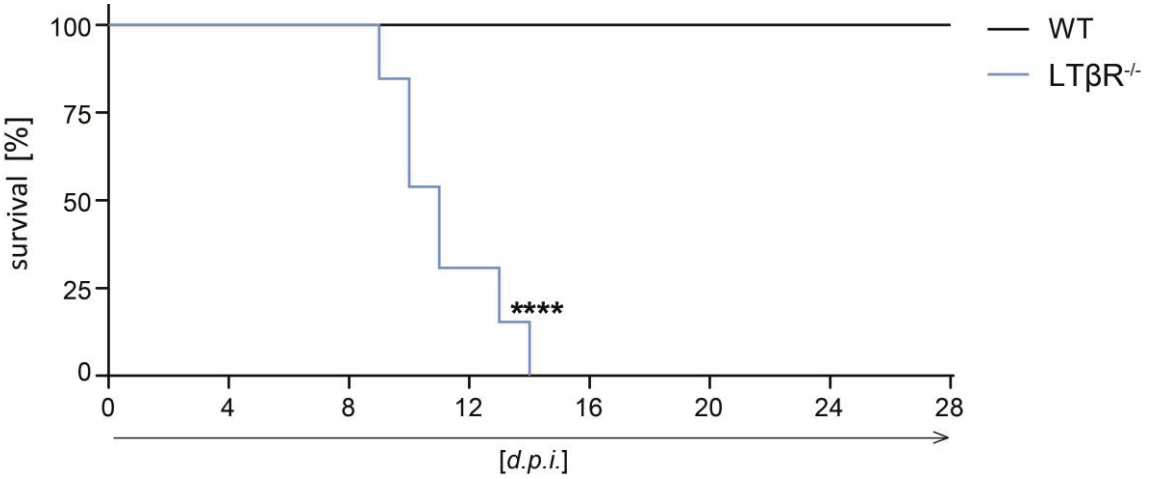
47. Coffman RL, Savelkoul HF, Lebman DA. Cytokine regulation of immunoglobulin isotype switching and expression. *Semin Immunol* 1989, **1**(1): 55-63.

48. Schluter D, Deckert M, Hof H, Frei K. 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* 2001, **69**(12): 7889-7893.

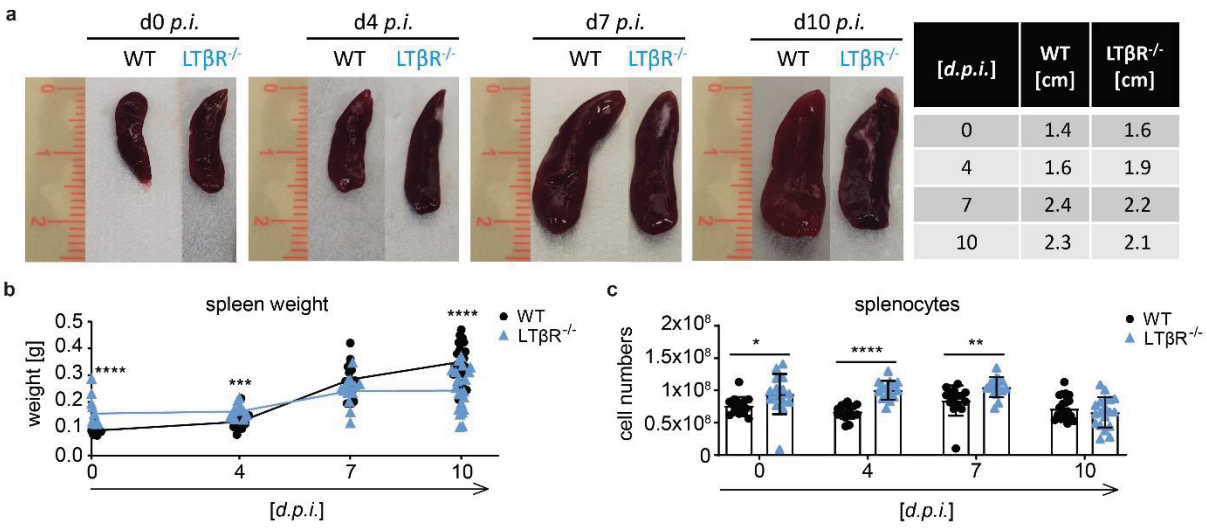
49. Virgen-Slane R, Correa RG, Ramezani-Rad P, Steen-Fuentes S, Detanico T, DiCandido MJ, et al. Cutting Edge: The RNA-Binding Protein Ewing Sarcoma Is a Novel Modulator of Lymphotoxin beta Receptor Signaling. *J Immunol* 2020, **204**(5): 1085-1090.

50. Chen TJ, Kotecha N. Cytobank: providing an analytics platform for community cytometry data analysis and collaboration. *Current topics in microbiology and immunology* 2014, **377**: 127-157.

Supplementary information

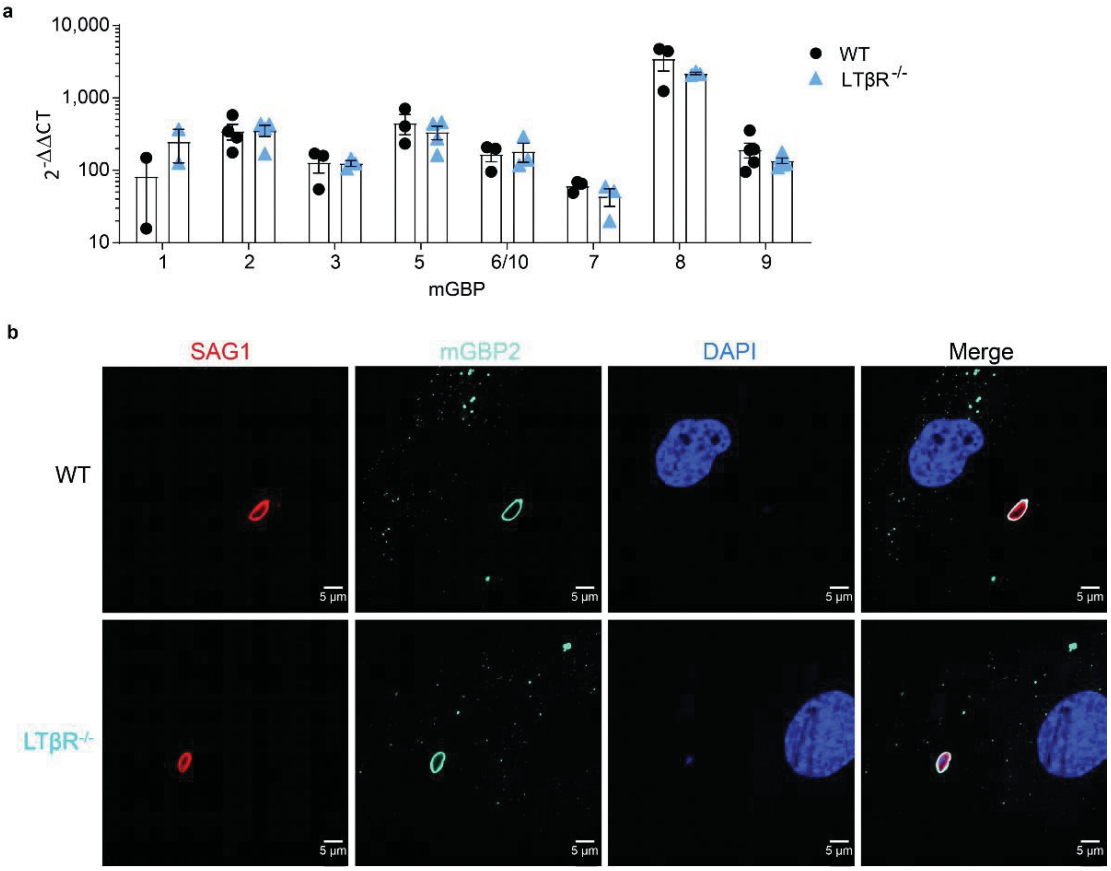


Supplementary Figure 1 | Increased susceptibility to *T. gondii*. Survival of *T. gondii* infected (ME49, 40 cysts, *i.p.*) WT; (n=15) and LTβR$^{-/-}$ (n=13) mice. Data shown represent three independent experiments. Unpaired two-tailed Student's *t*-test was used for statistical analysis. ****P<0.0001.

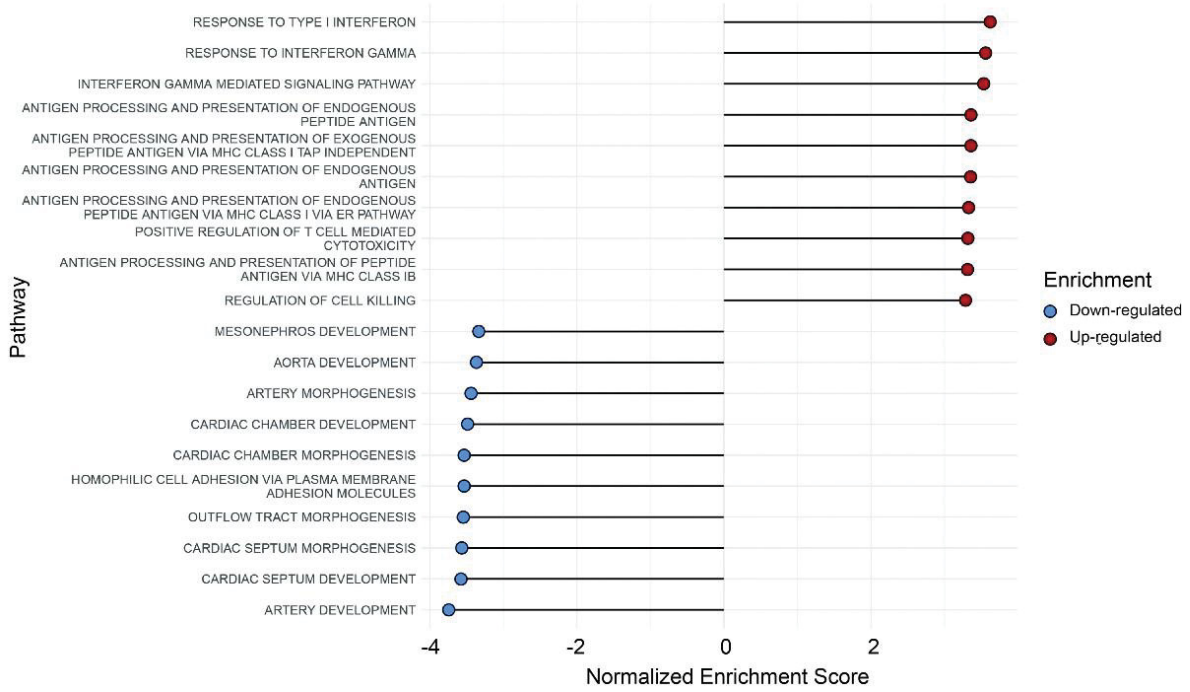


[d.p.i.]	WT [cm]	LTβR$^{-/-}$ [cm]
0	1.4	1.6
4	1.6	1.9
7	2.4	2.2
10	2.3	2.1

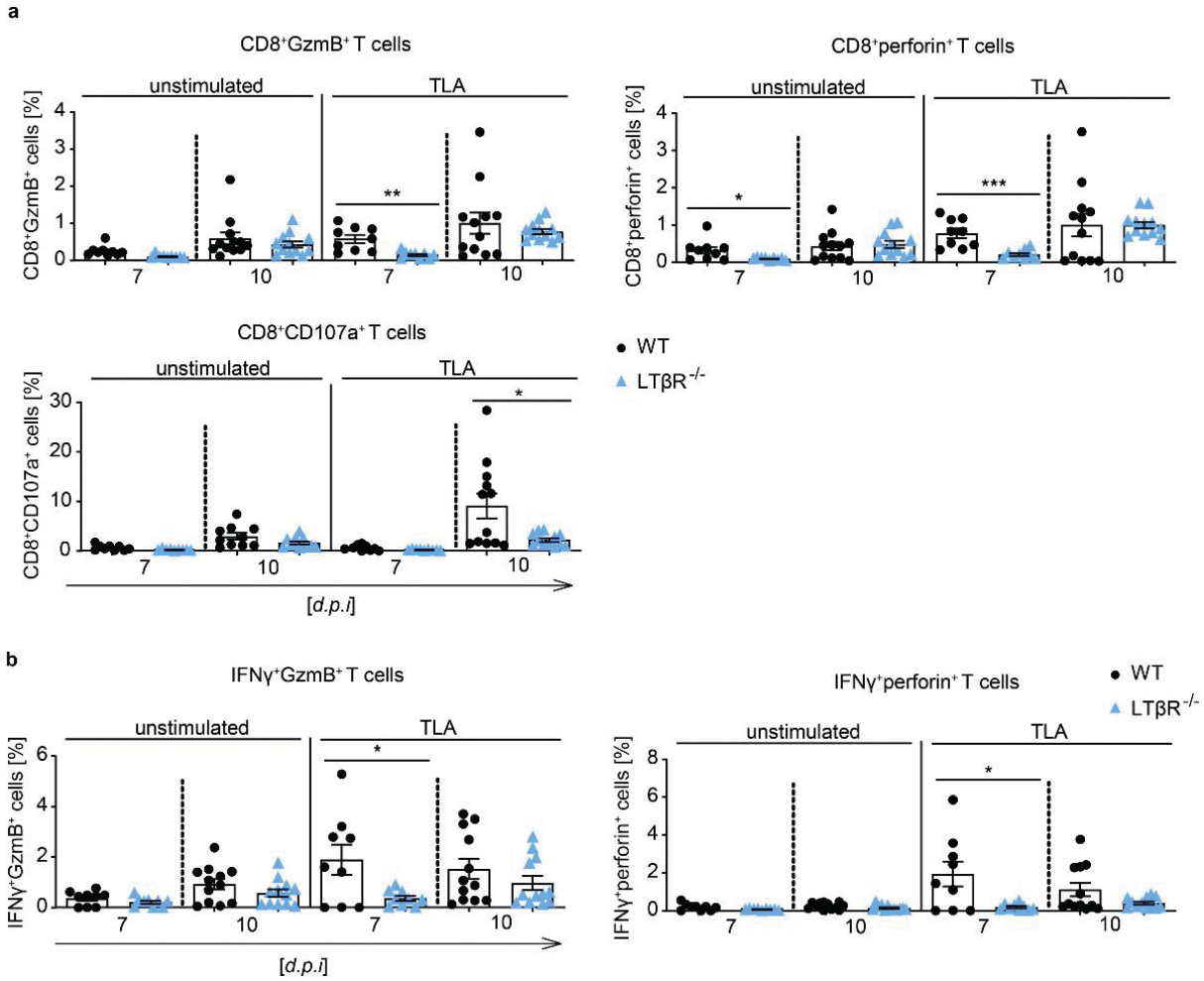
Supplementary Figure 2 | Decreased spleen weight but increased splenocyte numbers. a, Depiction of representative spleens from uninfected (d0) and *T. gondii* infected (ME49, 40 cysts, *i.p.*) WT and LTβR$^{-/-}$ mice (d0 – 7 *p.i.*: n=9, d10 *p.i.*:n≥9). Ruler indicated for scale [cm]. **b**, Spleen weight and **c**, absolute splenocyte numbers from uninfected (d0) 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. Unpaired two-tailed Student's *t*-test was used for statistical analysis. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.



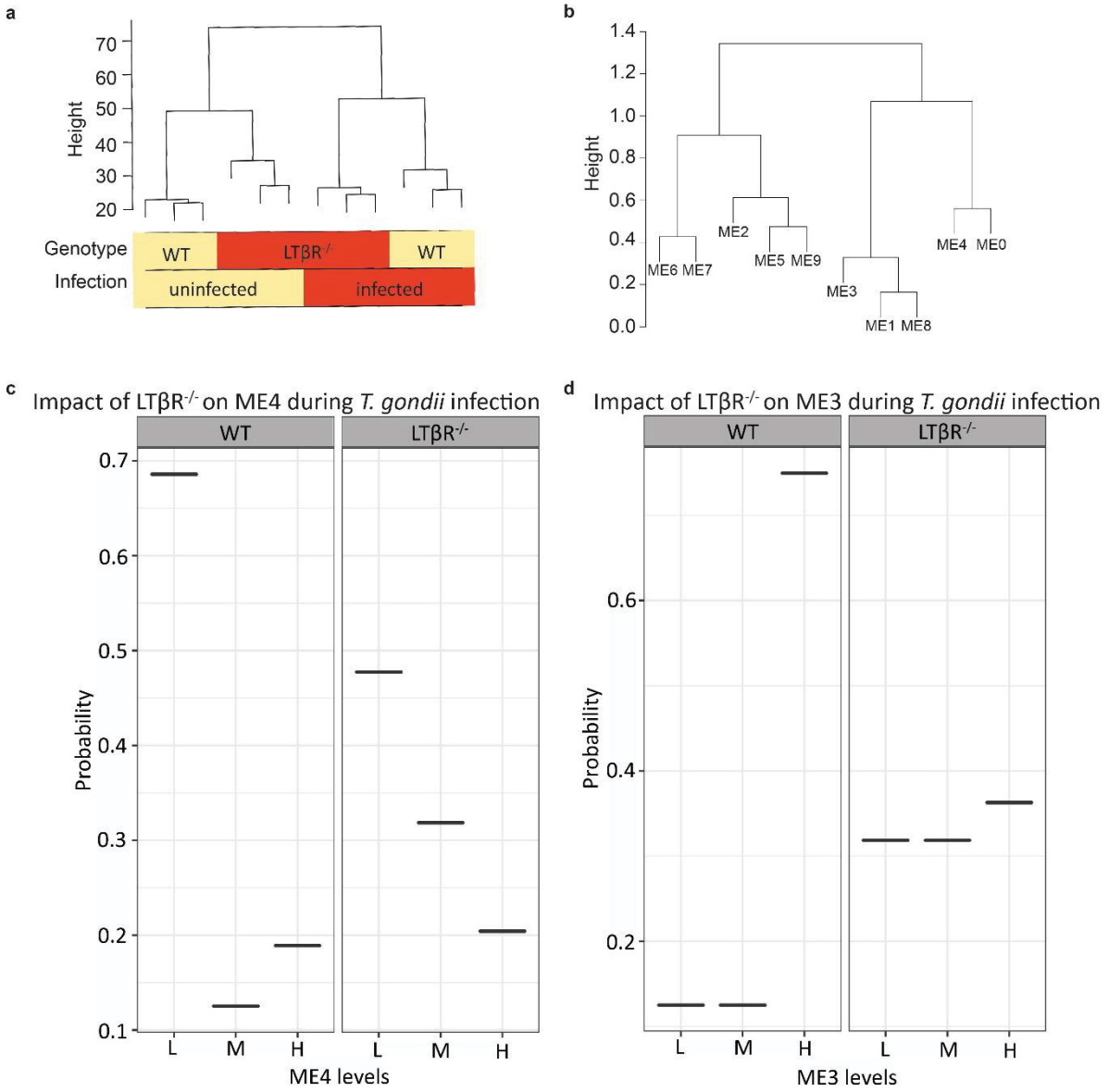
Supplementary Figure 3 | mGBP upregulation and recruitment. a, qRT-PCR analysis of mGBP mRNAs expression of uninfected WT and LTβR$^{-/-}$ MEFs stimulated with IFNγ (7.5 ng/ml) for 8h (all n=3, except for mGBP1 where n=2). Each symbol represents an individual techniqual replicate; columns represent mean values and error bars represent ± SEM. **b**, Representative immunofluorescence analysis of *T. gondii* tachyzoite (MOI 1:40) infected WT and LTβR$^{-/-}$ MEFs. Cells were prestimulated with IFNγ [7.5 ng/ml] for 16h before infected with *T. gondii* tachyzoites for 2h. *T. gondii* surface antigen SAG1 was visualized using a Cy3-conjugated and mGBP2 using an Alexa Fluor 633-conjugated secondary antibody for detection of mGBP2 recruitment towards the *T. gondii* PV. Cell nuclei were stained using DAPI. Data shown in **a** & **b** represent at least two independent experiments.



Supplementary Figure 4 | GSEA. a, 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.



Supplementary Figure 5 | T cell differentiation and degranulation. Intracellular staining of **a**, cytotoxic granule (granzyme B or perforin) containing and degranulating (CD107a) CD8⁺ T cells as well as **b**, IFNγ⁺ T cells of unstimulated and toxoplasma lysate antigen (TLA) *ex vivo* restimulated splenocytes from *T. gondii* infected (d7 and d10 *p.i.*) WT and LTβR$^{-/-}$ mice (d7: n≥6, d10: n≥10). Data shown represent at least two independent experiments; symbols represent individual animals, columns represent mean values and error bars represent ± SEM. Unpaired two-tailed Student's *t*-test was used for statistical analysis. *P<0.05, **P<0.01 and ***P<0.001.



Supplementary Figure 6 | Samples, modules and bayesian interference of key relationships from RNAseq analysis. a, Sample dendrogram (hierarchical clustering) and 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 KO 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: List of probes and primer sequences for amplification in qRT-PCR.

Gene	Probe [FAM]		Sequence [5' -> 3']
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
GTPBP1	75	fwd	GGTGCAGAGCAAAGATGATG
		rev	ATCTGGAATATCGGGCACAT
IFNγ	63	fwd	ATCTGGAGGAACTGGCAAAA
		rev	TTCAAGACTTCAAAGAGTCTGAGGTA
IFNβ	95	fwd	CAGGCAACCTTTAAGCATCAG
		rev	CCTTTGACCTTTCAAATGCAG
IL-12p40	27	fwd	GATTCAGACTCCAGGGGACA
		rev	TGGTTAGCTTCTGAGGACACATC
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	20	fwd	TGCCAACTTCCTCAGCTACA
		rev	GTGCACAGCAAAGTGATTGG
IDO1	2	fwd	GGGCTTCTTCCTCGTCTCTC
		rev	TGGATACAGTGGGGATTGCT
CD3	108	fwd	TCCCAACCCAGACTATGAGC
		rev	GCGATGTCTCTCCTATCTGTCA
CD4	99	fwd	AAAGAACTGGTTCGGCATGA
		rev	CGCTGACTCTCCCTCACTCT
CD8a	92	fwd	TGCTGTCCTTGATCATCACTCT
		rev	ACTAGCGGCCTGGGACAT
β-actin	106	fwd	TGACAGGATGCAGAAGGAGA
		rev	CGCTCAGGAGGAGCAATG
TgB1	TgB1*	fwd	GCTAAAGGCGTCATTGCTGTT
		rev	GGCGGAACCAACGGAAAT

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

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: List of primary antibodies used in immunoblot analyses.

Primary antibodies	Host species	Supplier
anti-Jak1 (D1T6W)	mouse	Cell Signaling Technology
anti-STAT1	rabbit	Cell Signaling Technology
anti-p-STAT (Tyr701)	rabbit	Cell Signaling Technology
anti-IRF-1 (D5E4) XP®	rabbit	Cell Signaling Technology
anti-STAT3 (79D7)	rabbit	Cell Signaling Technology
anti-p-STAT3 (Tyr705) (D3A7) XP®	rabbit	Cell Signaling Technology
anti-mGBP2	rabbit	Eurogentec
anti-mGBP7	rabbit	Eurogentec
anti-β-Actin (8H10D10)	mouse	Cell Signaling Technology
anti-GAPDH (14C10)	rabbit	Cell Signaling Technology

3.2 Lymphotoxin β receptor deficiency leads to dysregulation of anti-parasitic effector functions in *Toxoplasma gondii* infection

Authors

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

Published in

manuscript in preparation

Impact factor

-

Own contribution to this work

Overall: 80%

Conducted all of the following experiments:

T. gondii infection experiments, qRT-PCRs (mRNA expression and parasite load), ELISA, flow cytometry, immunoblot analysis, detection of *T. gondii* specific antibodies, serum transfer experiments

Other major contributions:

Designing the experiments, writing the manuscript, data analysis

Lymphotoxin β receptor deficiency leads to dysregulation of anti-parasitic effector functions in *Toxoplasma gondii* infection

Anne Tersteegen[1], Ursula R. Sorg[1], Richard Virgen-Slane[2], 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

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Abstract

LTβR mediated signaling is critically involved in the early immune response to intracellular pathogens such as *T. gondii*. In mice, LTβR signaling is essential to survive toxoplasmosis; however, the mechanisms responsible for the fatal loss of immunity in the absence of the LTβR are still not fully understood. Here, we provide evidence that LTβR deficiency dysregulates the immune responses in multiple organs including spleen, lung and brain tissue. LTβR$^{-/-}$ mice show altered numbers of immune cell populations in the spleen, increased IFNγ production in serum and fewer parasites in the brain. LTβR deficiency did not affect IFNγ protein expression in lung and brain tissue, but altered global gene expression in lung tissue and clearly affected IFNγ mediated gene as well as protein expression in lung and brain tissue. We also established the baseline parameters for an immune serum transfer model showing that transfer of *T. gondii* specific antibodies can rescue LTβR$^{-/-}$ mice after infection, demonstrating the importance of a function humoral immune response for the defence against *T. gondii*. These results provide new insights into the role of the LTβR in *T. gondii* infection and could provide valuable information for further studies exploring new therapeutic strategies, especially urgently needed vaccines.

Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan parasite that infects about one-third of the human population, causing a global health problem[1]. *T. gondii* can infect all nucleated cells of warm-blooded animals including humans which results in the disease toxoplasmosis[2]. During the acute phase of infection, *T. gondii* undergoes a fast lytic cycle where infectious tachyzoites replicate within infected cells and spread into various tissues via the blood and lymph[3]. Under pressure of the host immune response, tachyzoites convert to metabolically less active bradyzoites that reside in tissue cysts, primarily in the brain and muscle tissue, where they are able to evade the host immune system and establish chronic toxoplasmosis[4]. Acute toxoplasmosis usually causes no or mild flu-like symptoms, but can lead to life-threatening infections in immunocompromised individuals. In these patients, reactivation of chronic toxoplasmosis is also a potentially lethal medical complication. Primary infection of pregnant women can lead to congenital toxoplasmosis or cause miscarriage/stillbirth[2], as tachyzoites are able to pass through the placental barrier. The early, innate immune response to *T. gondii* is characterized by recognition of *T. gondii* associated molecules by immune cells, triggering cytokine production that in turn induces IFNγ production. IFNγ is typically produced by natural killer (NK) cells[5] and T cells[6, 7]. Whether other cell populations are also capable of producing interferon gamma has not yet been fully clarified as the available data is unclear. However, studies on the single cell level demonstrated that also dendritic cells (DCs)[8], monocytes[9], and macrophages[10] can produce IFNγ, which is known to play a vital role in *T. gondii* infection[11, 12, 13]. Induction of anti-parasite effector molecules[14, 15], such as production of indoleamine 2,3-Dioxygenase 1 (IDO)[16, 17] and inducible nitric oxide synthase (iNOS)[18, 19] as well as generation of reactive oxygen species (ROS)[20, 21] which suppress *T. gondii* replication[22, 23, 24], are important for restriction of parasite growth. In addition, murine guanylate-binding proteins (mGBPs) are known to be critical for controlling pathogens, as shown for *Listeria monocytogenes*[25], *Mycobacterium bovis BCG, Chlamydia muridarum*[26] and *Shigella flexneri*[27]. Studies show that core members of the tumor necrosis factor (TNF)/TNF receptor (TNFR) superfamily such as TNF and its receptors, lymphotoxin (LT) β and its receptor LTβR are crucial players in the immune response to *T. gondii*[28, 29, 30]. It is well documented that the LTβR plays an essential role in the organogenesis of peripheral lymphoid tissue, the maintenance of secondary peripheral organ structures as well as in innate immune responses to many pathogens[32, 33, 34]. LTβR deficiency (LTβR$^{-/-}$) results in a lack of lymph nodes and Peyer's patches, reduced NK- and DC cell numbers as well as impaired immunoglobulin (Ig) affinity maturation[35, 36]. Furthermore, LTβR$^{-/-}$ mice show conspicuous defects in the defence against *Listeria monocytogenes*, *Mycobacterium tuberculosis*[32], cytomegalovirus[37], Zika virus[38], and, *T. gondii*[28]. It has been shown that LTβR deficiency results in dramatically dysregulated IFNγ responses, impaired anti-parasite effector molecule expression and limited T cell functionality.

An abrogated *T. gondii* specific IgG response has been reported, highlighting the pivotal role of the LTβR also in the humoral immune response in *T. gondii* infection[39]. However, the pathophysiology causing the increased susceptibility of LTβR$^{-/-}$ mice to *T. gondii* infection is still not completely understood. Here, we demonstrate that LTβR deficiency dysregulates the numbers of immune cell populations in the spleen, increases IFNγ production in the serum and leads to a decrease of the parasite burden in the brain. It does not affect IFNγ protein expression in lung and brain tissue, but alters global gene expression in lung tissue and clearly affects IFNγ mediated gene as well as protein expression in lung and brain tissue. In addition, we provide initial data for developing an immune transfer model to assess the part that LTβR signaling plays in the humoral immune response to *T. gondii*. These results highlight the complex role of the LTβR in *T. gondii* infection and identify multiple starting points for further research that is necessary to gain new insights into the pathophysiology of toxoplasmosis and aid in developing much needed new treatment and prevention options, e.g. vaccination strategies, for human toxoplasmosis.

Results

LTβR deficiency dysregulates immune cell populations in the spleen. Initially, susceptibility of LTβR$^{-/-}$ mice to *T. gondii* infection was confirmed, the results showed that all mice succumbed to infection by day 14[39]. Interestingly, uninfected LTβR$^{-/-}$ mice showed increased spleen size compared to wildtype (WT) mice. During the course of infection, the spleen size of WT mice increases dramatically whereas those of LTβR$^{-/-}$ mice increases to a lesser extent[39]. This increase in WT animals is not only due to higher splenocyte numbers but also to inflammatory tissue swelling since splenocyte numbers in LTβR$^{-/-}$ animals are actually higher through day 7 post infection[39]. Since splenomegaly is also often present in patients who die due to *T. gondii* infection[40], splenocytes of uninfected (d0) and *T. gondii* infected (d4 – d10 *p.i.*) WT and LTβR$^{-/-}$ mice were analyzed via flow cytometry to determine their lineage (Fig. 1).

As total splenocyte numbers differed significantly between WT and LTβR$^{-/-}$ mice[39] absolute cell numbers were calculated (Fig. 1). Here, comparable absolute cell numbers of total DCs (CD11c^{+}MHCII^{all}), macrophages [total (MHCII^{int}CD11b^{+}), resident (F4/80^{+}Ly6C^{-}) and inflammatory (F4/80^{+}Ly6C^{+})], eosinophils (Ly6C^{int}SSC^{high}), neutrophils (Ly6C^{int}F4/80^{-}CD11b^{+}) and inflammatory monocytes (Ly6C^{+}F4/80^{+}CD11b^{+}) were detected in uninfected WT and LTβR$^{-/-}$ mice. In contrast, infected LTβR$^{-/-}$ mice exhibited significantly reduced numbers of these cell populations, with the exception of resident and inflammatory macrophages, on day 10 post infection. Furthermore, LTβR$^{-/-}$ mice showed reduced numbers of DCs on day 4 post infection as well as significantly reduced numbers of classical (c) DCs (CD11c^{high}MHCII^{+}) and plasmacytoid (p) DCs (BST2^{+}CD11b^{-}CD11c^{int}) on all days analyzed. Even though WT and

LTβR$^{-/-}$ mice possess comparable numbers of macrophages, the number of tissue resident macrophages was significantly increased in LTβR$^{-/-}$ mice on day 4 post infection. While WT animals showed increased numbers of eosinophils and neutrophils on day 10 post infection, their numbers did not increase in LTβR$^{-/-}$ mice during infection.

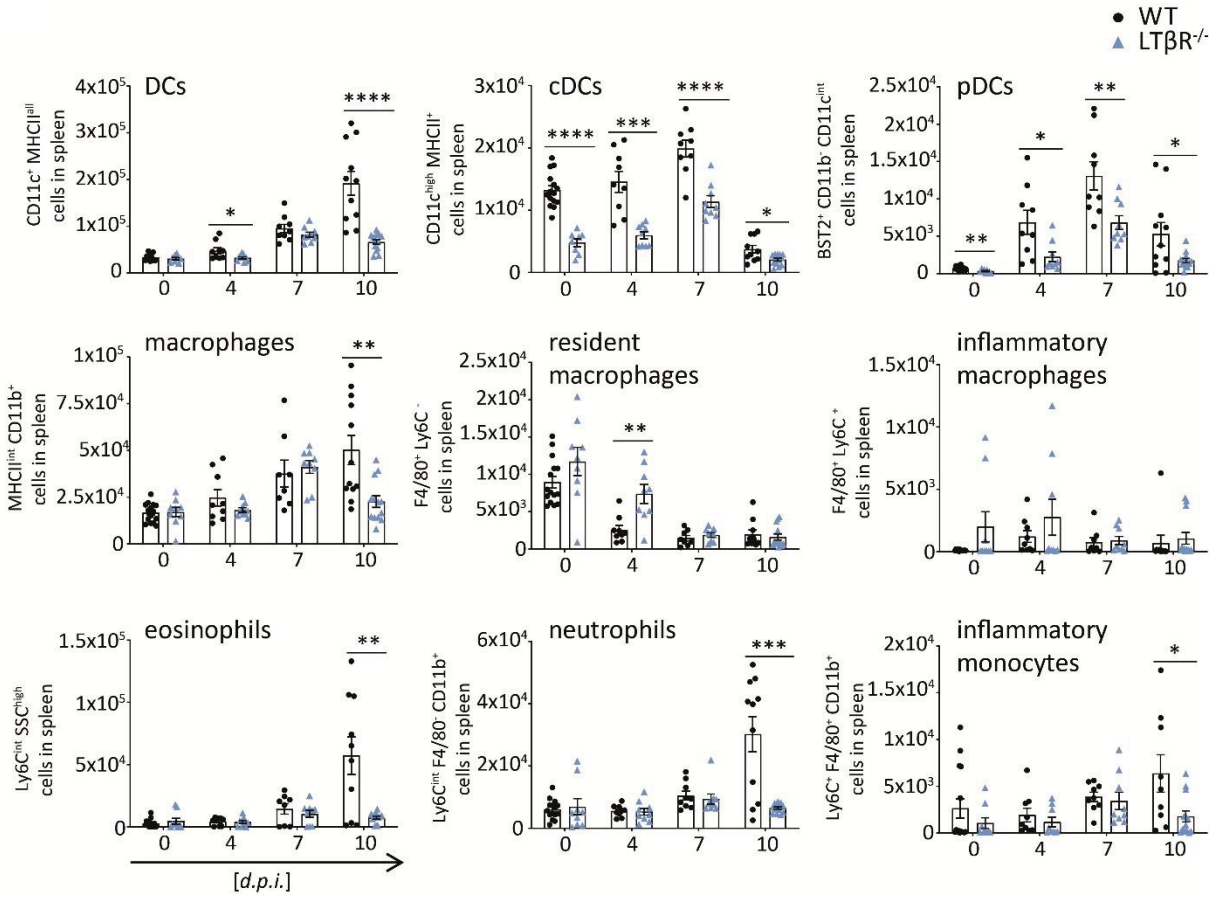

Fig. 1 | Dysregulated immune cell numbers in spleens of LTβR$^{-/-}$ mice. Absolute cell numbers of DCs (CD11c^{+}MHCII^{all}), cDCs (CD11c^{high}MHCII^{+}), pDCs (BST2^{+}CD11b^{-}CD11c^{int}), macrophages (MHCII^{int}CD11b^{+}), resident (F4/80^{+}Ly6C^{-}) and inflammatory (F4/80^{+}Ly6C^{+}) macrophages, eosinophils (Ly6C^{int}SSC^{high}), neutrophils (Ly6C^{int}F4/80^{-}CD11b^{+}) and inflammatory monocytes (Ly6C^{+}F4/80^{+}CD11b^{+}) in spleens of uninfected (d0) and *T. gondii* infected WT and LTβR$^{-/-}$ mice (d0 – 10 *p.i.*: n≥9) determined via flow cytometry. Data shown in **b** are from at least three independent experiments; symbols represent individual animals, bars show mean values ± SEM. Unpaired two-tailed Student's *t*-test was used for statistical analysis. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Unbiased, multidimensional tSNE clustering analysis of representative uninfected (d0) and *T. gondii* infected (d7 *p.i.*) WT und LTβR$^{-/-}$ mice was performed (Fig. 2). Here, comparable frequencies of CD11c^{+}MHCII^{all} (all DCs) and BST1^{+}CD11b^{-}CD11c^{int} (pDCs) cells (Fig. 2a) as well as similar frequencies of MHCII^{int}CD11b^{+} (macrophages) and Ly6C^{int}F4/80^{-}C11b^{+} (neutrophils) cells (Fig. 2b) were detected in uninfected and infected WT and LTβR$^{-/-}$ mice. Furthermore, tSNE analysis showed slightly reduced frequencies of CD11c^{high}MHCII^{+} (cDCs) cells in infected and uninfected LTβR$^{-/-}$ mice (Fig. 2a) as well as reduced frequencies of Ly6C^{int}SSC^{high} (eosinophils) cells (d7 *p.i.*; Fig. 2b) compared to WT mice.

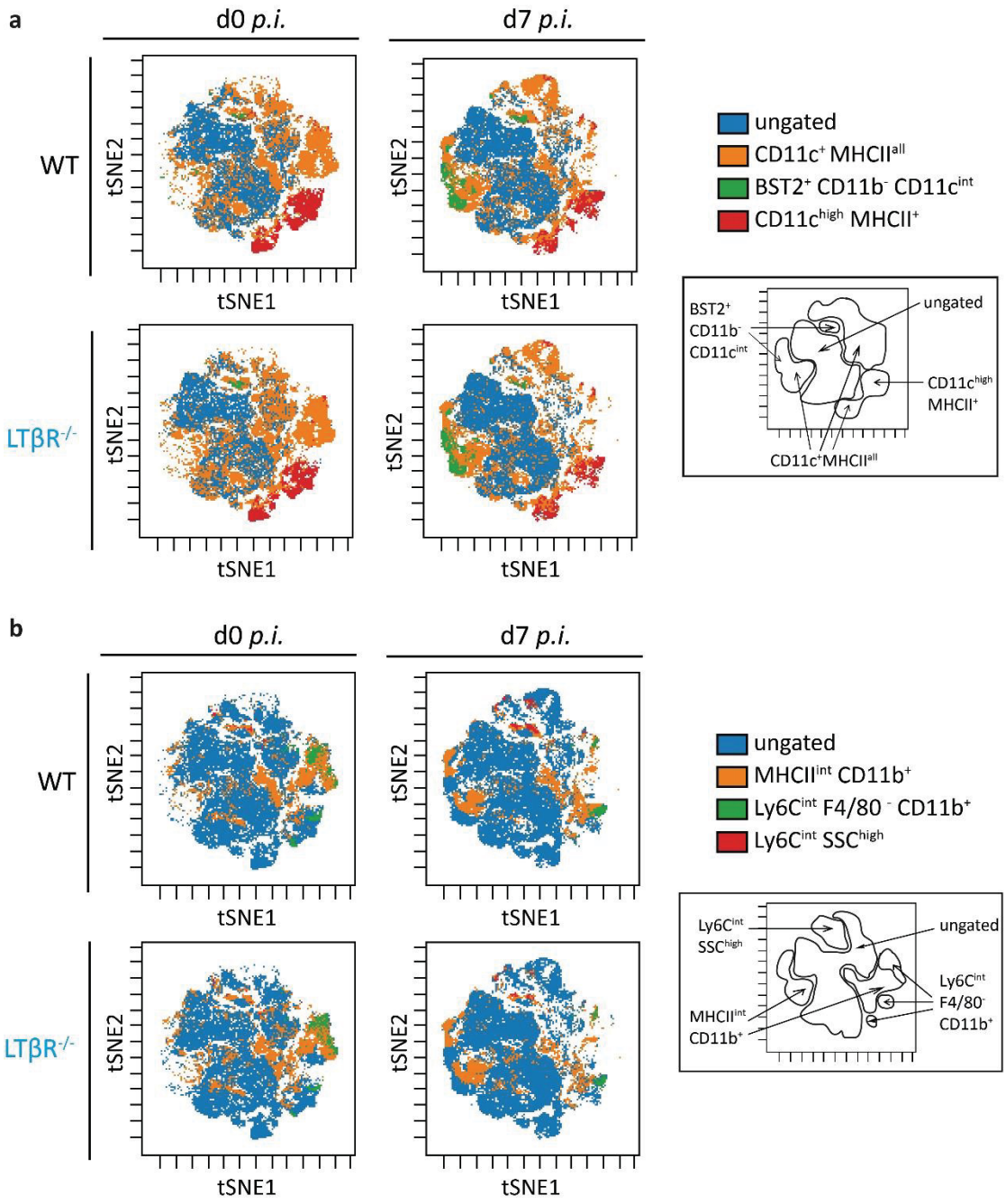

Fig. 2 | tSNE analysis of splenocytes. a and b, Representative tSNE plots from splenocytes of uninfected (d0) and *T. gondii* infected (d7 *p.i.*) WT and LTβR$^{-/-}$ mice (n≥9). tSNE analysis was performed on pregated non T and non B cells and clustered populations were identified using the indicated markers. Uncolored schemes of clusters are included for identification of cell populations. Data shown represent one of at least three independent experiments.

Due to the observation that LTβR deficiency influences various immune cell populations in *T. gondii* infected mice T cell subsets were further analyzed for memory, naïve and effector CD4$^+$ (Fig. 3a) and CD8$^+$ (Fig. 3b) T cells. To this end, splenocytes of infected mice (d7 and d10 *p.i.*) remained unstimulated or were restimulated *ex vivo* with toxoplasma lysate antigen (TLA) before analysis using flow cytometry. LTβR$^{-/-}$ mice compared to WT mice showed increased numbers of memory and naïve CD4$^+$ T cells on day 10 post infection under unstimulated conditions and increased naïve CD4$^+$ T cells following TLA restimulation.

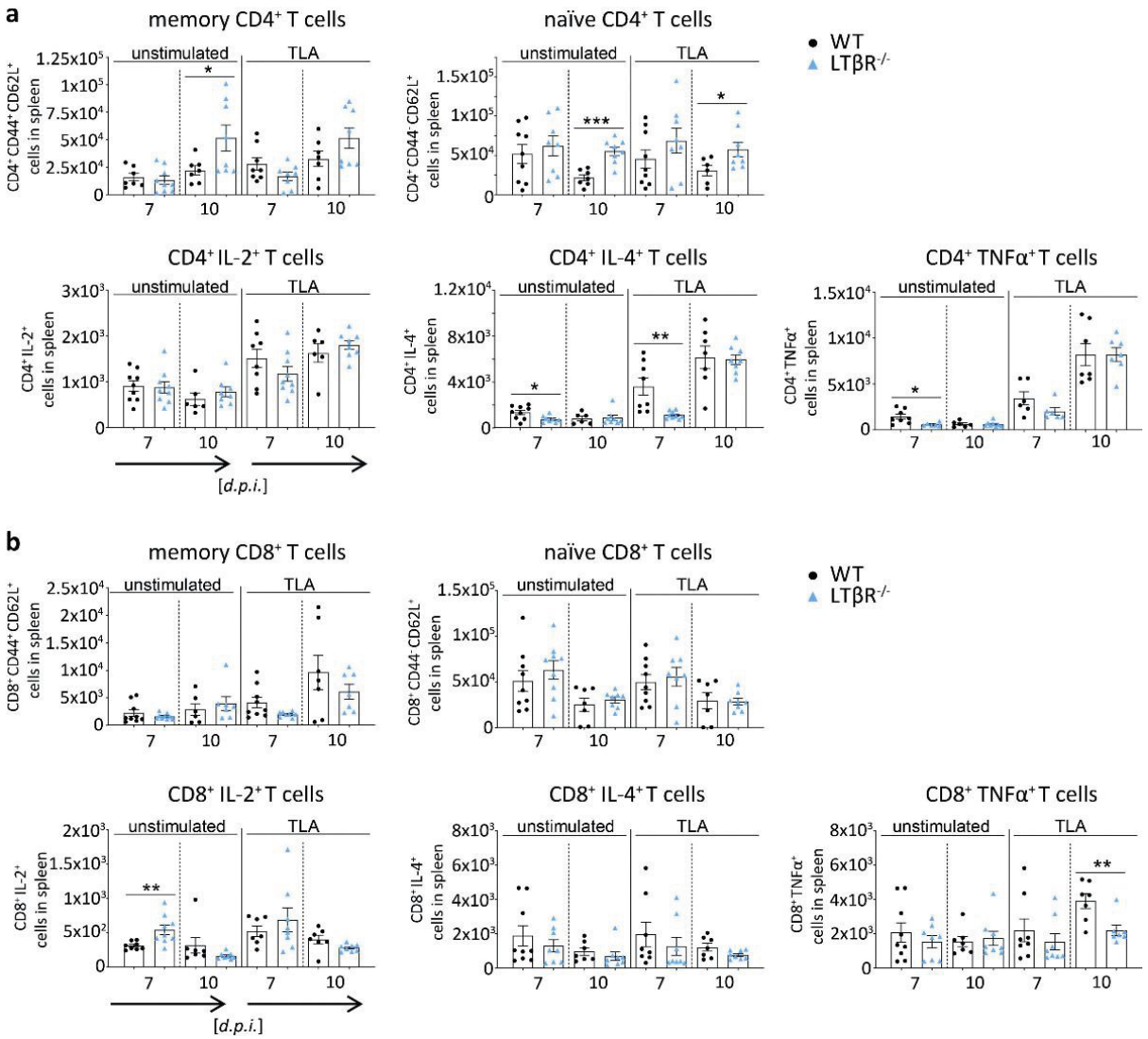

Fig. 3 | Altered T cell subsets in spleens of LTβR$^{-/-}$ mice. Intracellular staining of T cells of unstimulated and toxoplasma lysate antigen (TLA) restimulated splenocytes of *T. gondii* infected (d7 and 10 *p.i.:* n≥5) WT and LTβR$^{-/-}$ mice. **a**, Absolute cell numbers of memory and naïve CD4$^+$ T cells as well as IL2$^+$, IL4$^+$ and TNFα$^+$ CD4$^+$ T cells. **b**, Absolute cell numbers of memory and naïve CD8$^+$ T cells as well as IL2$^+$, IL4$^+$ and TNFα$^+$ CD8$^+$ T cells. All data shown are from at least two independent experiments; symbols represent individual animals, bars show mean values ± SEM. Unpaired two-tailed Student's *t*-test was used for statistical analysis. *P<0.05 and **P<0.01.

CD4$^+$IL-2$^+$ T cell numbers were comparable, whereas reduced numbers of CD4$^+$IL-4$^+$ and CD4$^+$TNFα$^+$ effector T cells were detected in LTβR$^{-/-}$ mice on day 7 post infection compared to WT mice (Fig. 3a). Comparable numbers of memory, naïve and IL-4$^+$ and CD8$^+$ T cells were detected in WT and LTβR$^{-/-}$ mice. LTβR$^{-/-}$ mice showed increased numbers of unstimulated CD8$^+$IL-2$^+$ (d7 *p.i.*) in unstimulated splenocytes and decreased numbers of CD8$^+$TNFα$^+$ effector T cells (d10 *p.i.*) in TLA restimulated splenocytes compared to WT mice.

LTβR deficiency does not affect IFNγ protein expression in lung tissue. Since *T. gondii* replicates during the acute phase of infection within the lung, IFNγ protein expression was

analyzed via ELISA (Fig. 4a) next. Comparable amounts of IFNγ were detected in lung tissue of uninfected (d0) and *T. gondii* infected (d4 – d10 *p.i.*) WT and LTβR$^{-/-}$ mice. IFNγ production increased during infection until day 7 post infection in both cohorts. Because suppressor of cytokine signaling (SOCS) proteins, especially SOCS1 and SOCS3, are involved in inhibition of IFNγ mediated JAK-STAT signaling by inhibiting JAK phosphorylation of signal transducer and activators of transcription (STAT), mRNA expression of both genes was analyzed via qRT-PCR (Fig. 4b)[41, 42]. LTβR$^{-/-}$ mice compared to WT mice showed significantly increased SOCS1 and SOCS3 mRNA expression in lung tissue of uninfected (d0) animals as well as increased SOCS1 expression in *T. gondii* infected LTβR$^{-/-}$ mice on day 10 post infection.

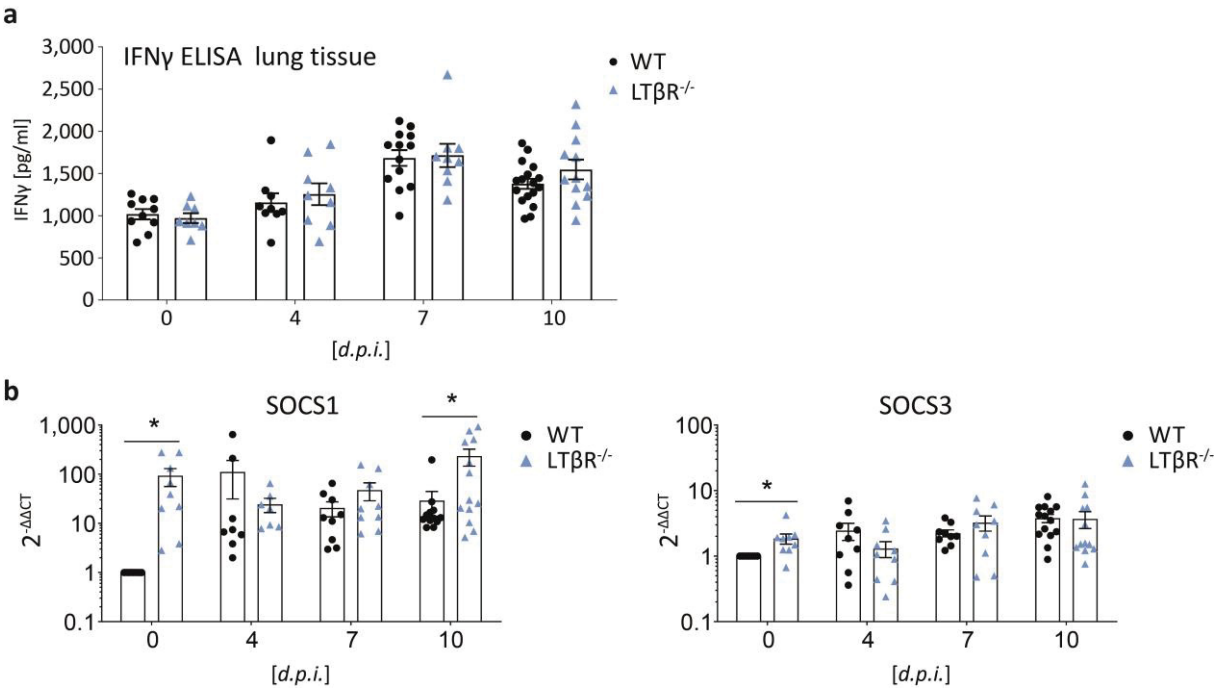

Fig. 4 | IFNγ response in lungs of LTβR$^{-/-}$ mice. a, IFNγ ELISA (d0 – 7 *p.i.*: n≥8, d10 *p.i.*: n≥12) and **b**, qRT-PCR analysis of SOCS family members (d0 – 7 *p.i.*: n=9, d10 *p.i.*: n≥12) in lung tissue of uninfected (d0) and *T. gondii* infected WT and LTβR$^{-/-}$ mice. Data shown are from four independent experiments; symbols represent individual animals, bars show mean values ± SEM. Unpaired two-tailed Student's *t*-test was used for statistical analysis. *P<0.05.

LTβR deficiency alters global gene expression in lung tissue. Next, RNAseq analysis of lung tissue of uninfected (d0) and *T. gondii* infected (d7 *p.i.*) WT and LTβR$^{-/-}$ mice was performed. Figure 5a shows a heatmap depicting the top 40 differentially expressed genes. On the one hand it indicates an increased activation status of uninfected LTβR$^{-/-}$ mice compared to the uninfected WT mice and on the other hand it highlights a lack of global upregulation in LTβR$^{-/-}$ mice after *T. gondii* infection, which can be clearly observed in infected WT mice. This is reflected on a global level when referring to the corresponding 3D principal component analysis (PCA, Fig. 5b) that clearly identified four different clusters: uninfected WT

(green) and LTβR$^{-/-}$ (orange) mice as well as *T. gondii* infected WT (pink) and LTβR$^{-/-}$ (purple) mice. The arrangement of the clusters demonstrates that gene expression of WT and LTβR$^{-/-}$ differs as widely in uninfected WT vs. LTβR$^{-/-}$ as in infected WT vs. LTβR$^{-/-}$ mice.

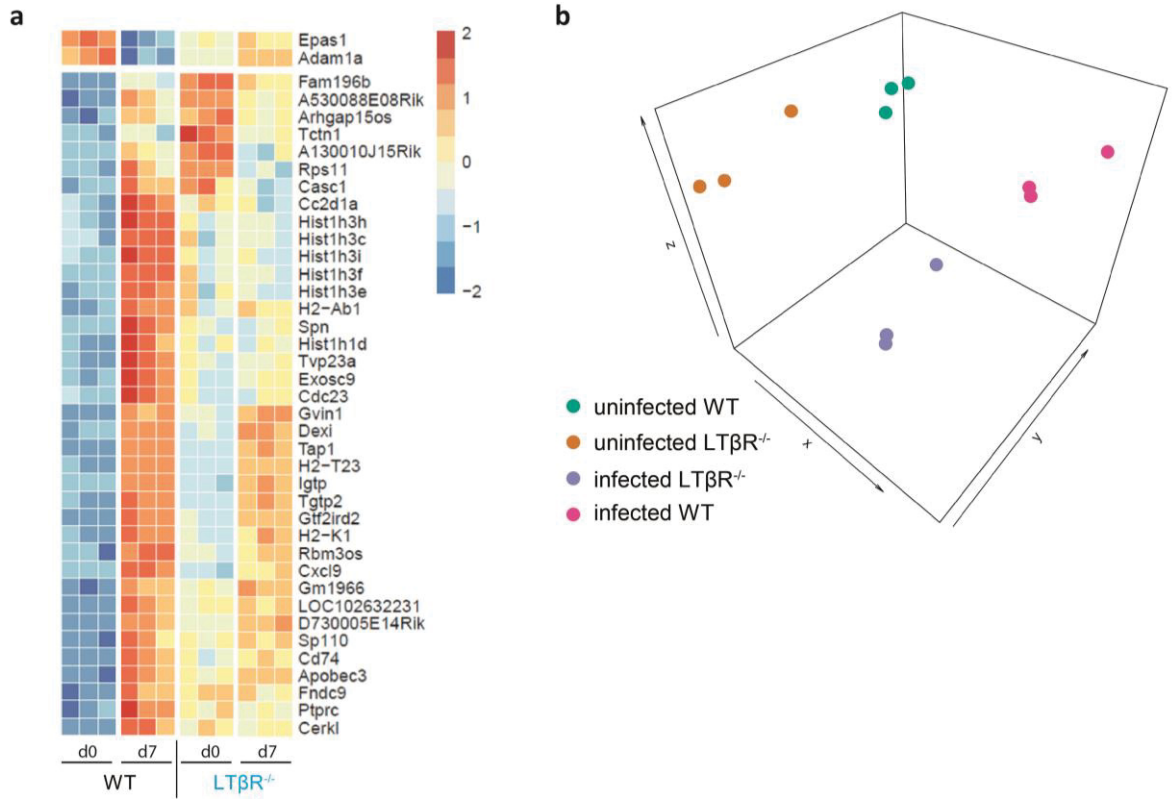

Fig. 5 | Altered gene activation status in lungs of LTβR$^{-/-}$ mice. a, heat map of the top 40 differentially expressed genes and **b**, 3D principal component analysis (PCA) based on RNAseq analysis of lung tissue of uninfected (d0) and *T. gondii* infected (d7 *p.i.*) WT and LTβR$^{-/-}$ mice (n=3). DEseq2 was used to test for differential expression, “Wald” test and an adjusted p-value of 0.1 were used to determine significance.

To further investigate altered gene expression in lung tissue RNAseq data was analyzed, visualized as a volcano plot and gene set enrichment analysis (GSEA) was performed.

This analysis (Fig. 6a) reports differential expression of genes due to LTβR deficiency alone. Data of lung tissue from uninfected and *T. gondii* infected (d7 *p.i.*) LTβR$^{-/-}$ mice was correlated to data from uninfected WT mice to identify differential basal gene expression. Data from infected LTβR$^{-/-}$ mice was included to find genes that are differential expressed due to LTβR deficiency, but do not respond to infection.

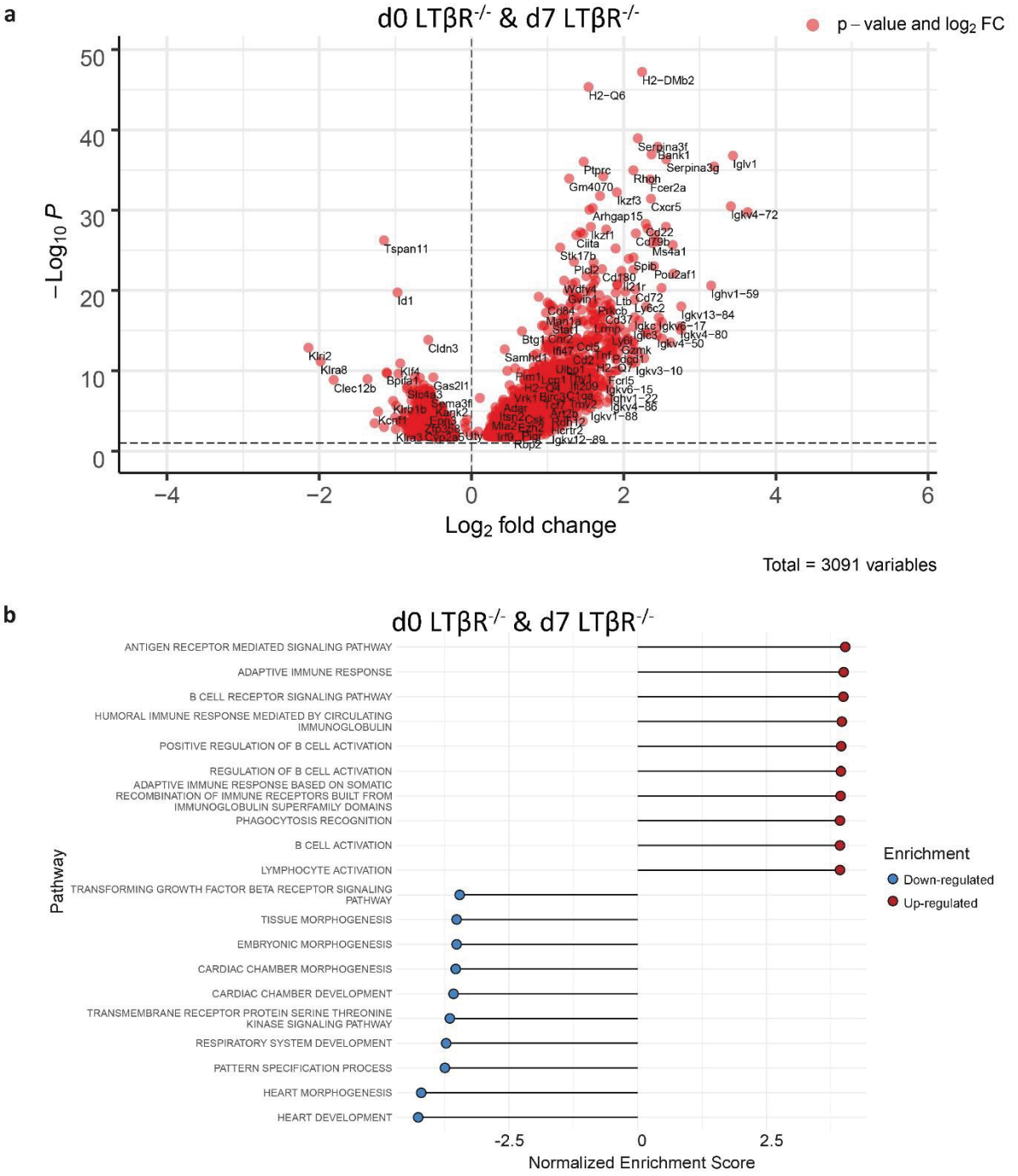

Fig. 6| Altered gene expression in lungs of uninfected and infected LTβR$^{-/-}$ mice. Analysis of RNAseq data of lung tissue of uninfected (d0) and infected (d7 *p.i.*) LTβR$^{-/-}$ mice (n=3) depicted as **a,** volcano plot and **b**, GSEA analysis. Dashed horizontal black line represents p=0.05. For RNAseq data mouse transcripts from pseudogenes or with retained introns were excluded prior to conversion to gene counts. DEseq2 was used to test for differential expression and GO (Biological Process) molecular signatures were obtained using the msigdr package in R. Gene-level stats for each differential expression contrast were tested for geneset enrichment using the fgsea package, with an adjusted p-value of 0.1.

Particularly immunoglobulin (Ig) chain variants are massively upregulated in LTβR$^{-/-}$ mice. This is confirmed in the GSEA where LTβR$^{-/-}$ mice show up-regulation in pathways such as “antigen receptor mediated signaling”, “B cell receptor signaling” and “humoral immune response mediated by circulating immunoglobulin” which include Ig chain variants (Fig. 6b).

Preliminary analysis of transcriptome data (Supplementary Fig. 1a), illustrating differentially expressed genes in *T. gondii* infected (d7 *p.i.*) LTβR$^{-/-}$ mice compared to uninfected LTβR$^{-/-}$ mice, also showed a global lack of transcript up-regulation in infected LTβR$^{-/-}$ mice in contrast to uninfected animals. Specifically, expression of histones, IFNγ-regulated genes and cytokines seemed to be affected. Also the corresponding GSEA analysis (Supplementary Fig. 1b) of LTβR$^{-/-}$ animals on day 7 post infection showed that they do not up-regulate genes involved in "response to interferon gamma*"* and "interferon gamma mediated signaling pathway".

LTβR deficiency affects expression of genes involved in IFNγ signaling in lung tissue. It is known that IFNγ-regulated effector molecules are pivotal in *T. gondii* containment[43]. Our results demonstrate that LTβR deficiency does not only affect IFNγ expression itself but also IFNγ related signaling pathways. In order to investigate a possible interaction between LTβR and IFNγ signaling in uninfected mice the impact of LTβR deficiency on the IFNγ signaling network was assessed via pathway analysis using MetaCore. This form of analysis depicts a known network of protein interactions (a reference guide describing the different symbols is provided in Supplementary Figure 2 & 3) and superimposes the results of an RNAseq analysis. Genes with significant differential expression in LTβR$^{-/-}$ animals are annotated with a small thermometer symbol, red for increased, blue for decreased expression. Figures 7 depicts the role of IFNγ in macrophage activation. Also IFNγ signaling via MAPK, via JAK-STAT & PLCγ and via PI3K & NFκB is shown (Fig. 8-10). While the IFNγ signaling network is too complex to make it feasible to describe all possible interactions comprehensively, a few salient points can be made:

LTβR deficiency leads to a slight increase in basal IFNγ expression that could modulate signaling via the IFNγR (Fig. 7). Within the macrophage activation pathway network this receptor binding could increase and activate JAK1 and JAK2 expression that in turn could result in increased STAT1 activation, transcriptional expression and phosphorylation. Enhanced STAT1 expression would then explain the increased expression of cytokines (IL-12β, TNF), chemokines (IP10, CCL2, CCL5) and the surface protein CD40, a member of the TNF/TNFR superfamily. This would (positively) influence, either directly or more indirectly, the inflammatory response (black arrows). The depicted increased expression of IRF-8, c-Rel and PU.1 genes could be responsible for the increase in gp91-phox and p67-phox expression, two molecules known to be involved in the synthesis of reactive oxygen species (blue arrows).

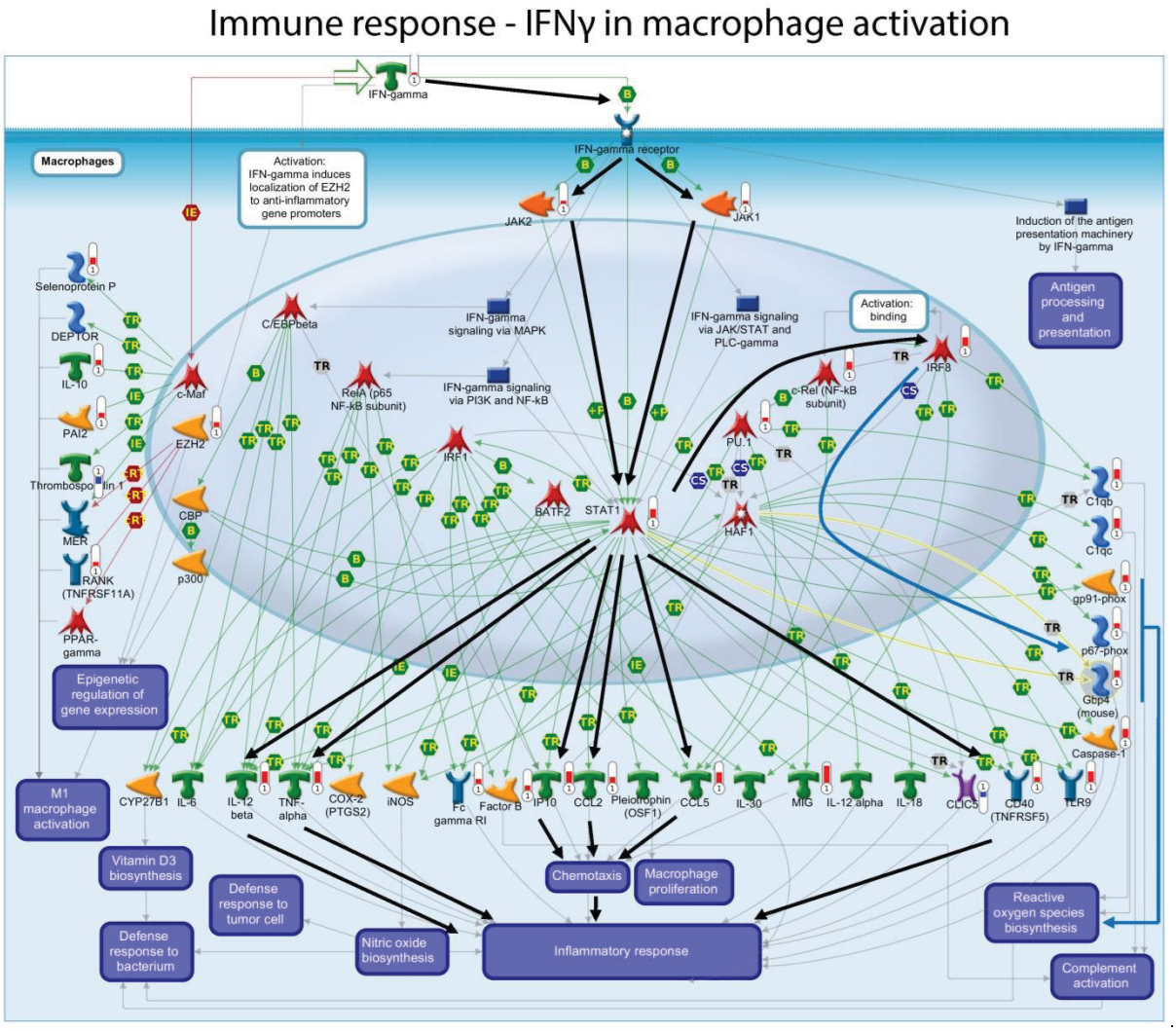

Fig. 7 | Impact of LTβR deficiency on IFNγ signaling in macrophage activation. The impact of LTβR deficiency on IFNγ pathways is visualized using MetaCore. Lung tissue of uninfected (d0) WT and LTβR$^{-/-}$ mice was used for RNA sequencing. Significant differential gene expression data is superimposed onto the map and visualized as thermometer-like symbols. Upward pointing thermometers are depicted in red and describe upregulated gene expression and downward pointing (blue) thermometers describe downregulated gene expression. Colored lines indicate activation (green), inhibition (red) or unspecified (grey) interactions between the molecules. Interactions that are in the network base, but not the network generated by integrated gene expression data are highlighted in yellow. DEseq2 was used to test for differential expression, "Wald" test and an adjusted p-value of 0.1 were used to determine significance. The object shapes correspond to specific molecule types and all symbols and arrows etc. are described in supplementary figures 2 & 3. The most strongly affected pathways are marked by bold arrows.

Increased IFNγ expression that could modulate signaling via the IFNγR could also increase and activate p38 MAPK expression (Fig. 8) in LTβR$^{-/-}$ cells via increased activation and phosphorylation via various kinases. p38 MAPK expression activates MAPKAPK2 that inhibits downstream phosphorylation, thus possibly increasing TNF and IP10 expression. This increased expression could exacerbate the inflammatory response in LTβR$^{-/-}$ mice.

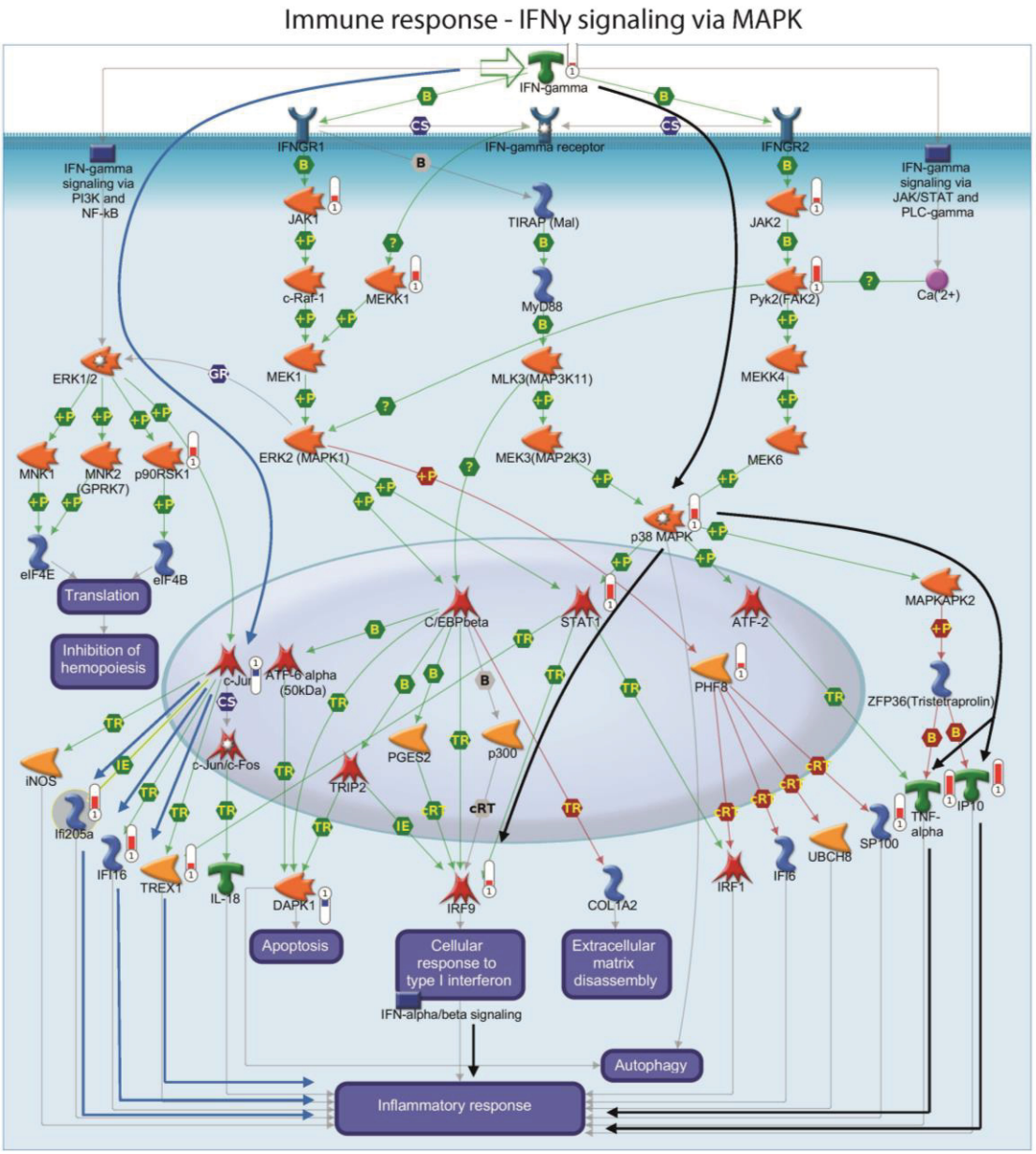

Fig. 8| Impact of LTβR deficiency on IFNγ signaling via MAPK pathway. The impact of LTβR deficiency on IFNγ pathways is visualized using MetaCore. Lung tissue of uninfected (d0) WT and LTβR$^{-/-}$ mice was used for RNA sequencing. Significant differential gene expression data is superimposed onto the map and visualized as thermometer-like symbols. Upward pointing thermometers are depicted in red and describe upregulated gene expression and downward pointing (blue) thermometers describe downregulated gene expression. Colored lines indicate activation (green), inhibition (red) or unspecified (grey) interactions between the molecules. Interactions that are in the network base, but not the network generated by integrated gene expression data are highlighted in yellow. DEseq2 was used to test for differential expression, "Wald" test and an adjusted p-value of 0.1 were used to determine significance. The object shapes correspond to specific molecule types and all symbols and arrows etc. are described in supplementary figures 2 & 3. The most strongly affected pathways are marked by bold arrows.

In addition, the increased expression of IRF-9, possibly due to increased STAT1 activation and expression, would influence the cellular response to type I interferons and ultimately also contribute to inflammatory processes (black arrows). c-Jun mediated expression of Ifi205a, a transcriptional regulator, IFI16, a transcriptional repressor and TREX1, a DNA binding protein,

could also further modulate the basal inflammatory activation status (blue arrows) as previously described on the basis of the heatmap (Fig. 5a). Next, the impact of LTβR deficiency in IFNγ signaling via JAK-STAT and PLCγ was assessed (Fig. 9).

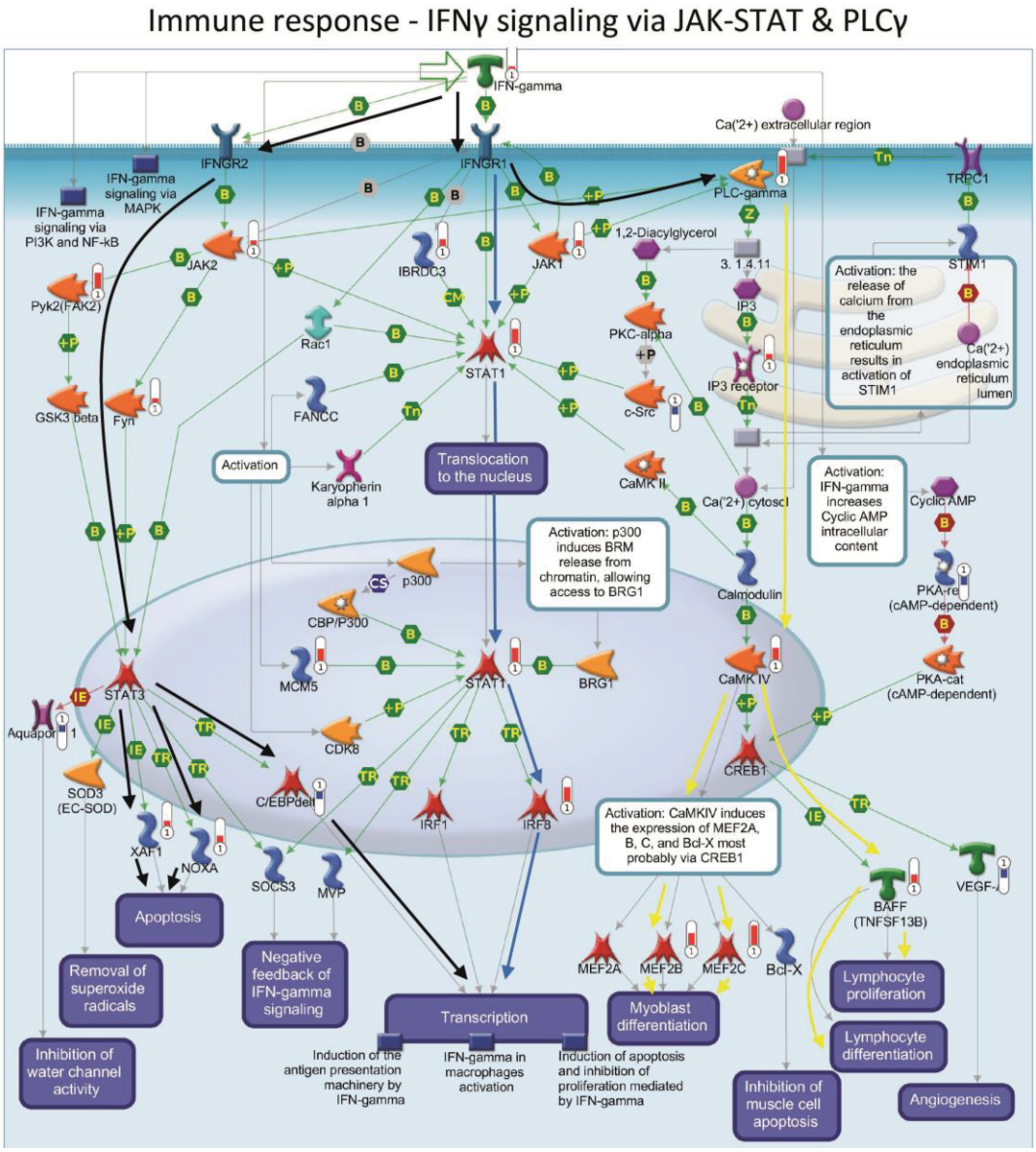

Fig. 9 | Impact of LTβR deficiency on IFNγ signaling via JAK-STAT & PLCγ pathways. The impact of LTβR deficiency on IFNγ pathways is visualized using MetaCore. Lung tissue of uninfected (d0) WT and LTβR$^{-/-}$ mice was used for RNA sequencing. Significant differential gene expression data is superimposed onto the map and visualized as thermometer-like symbols. Upward pointing thermometers are depicted in red and describe upregulated gene expression and downward pointing (blue) thermometers describe downregulated gene expression. Colored lines indicate activation (green), inhibition (red) or unspecified (grey) interactions between the molecules. DEseq2 was used to test for differential expression, "Wald" test and an adjusted p-value of 0.1 were used to determine significance. The object shapes correspond to specific molecule types and all symbols and arrows etc. are described in supplementary figures 2 & 3. The most strongly affected pathways are marked by bold arrows.

Increased JAK2 gene expression in lungs of LTβR$^{-/-}$ mice could induce increased FAK2 and Fyn expression that in turn could indirectly result in increased XAF1 and NOXA as well as

decreased C/EBPδ expression, possibly affecting apoptosis and IFNγ induced transcription, respectively (black arrows).

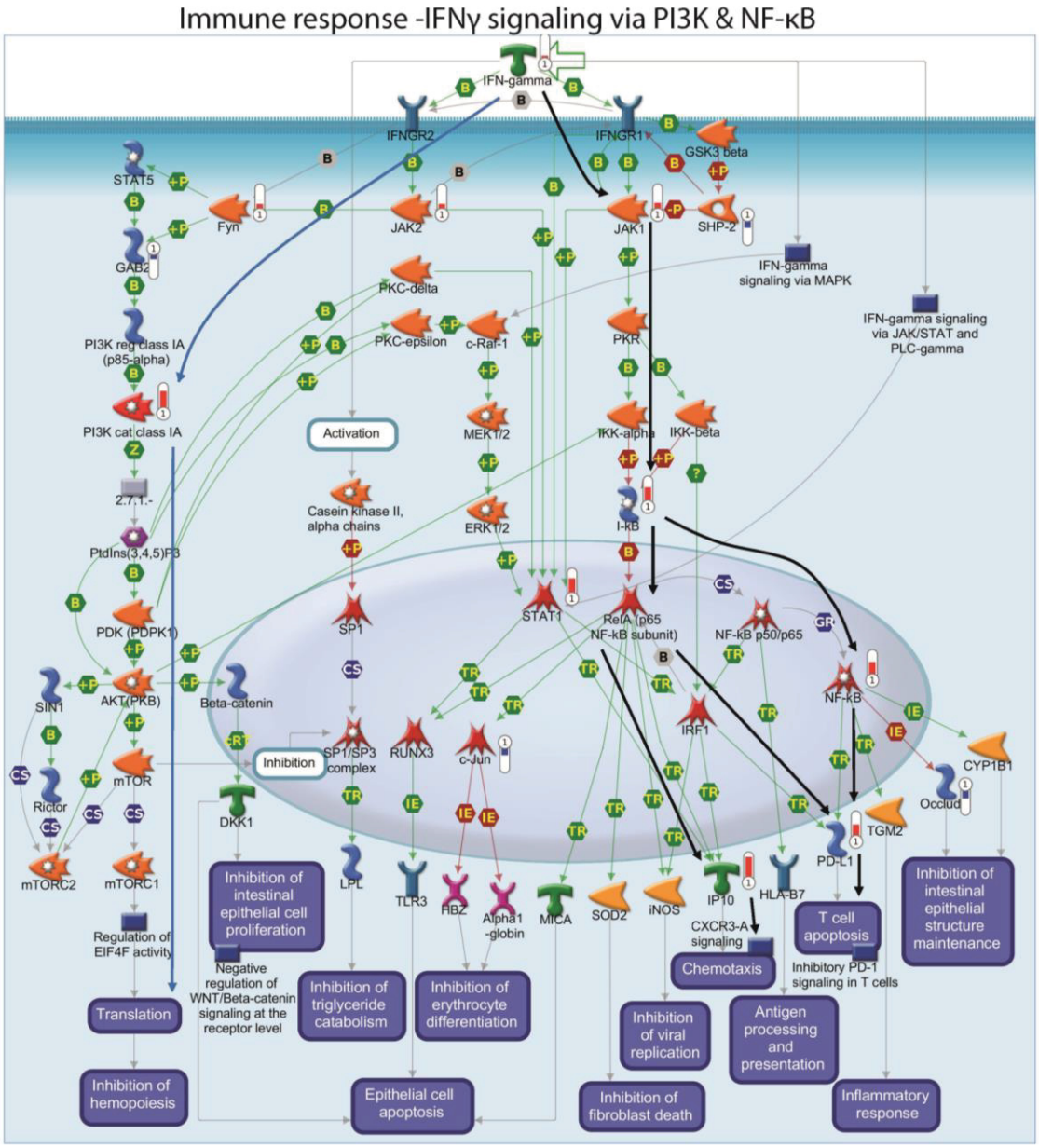

Fig. 10 | Impact of LTβR deficiency on IFNγ signaling via PI3K & NFκB pathways. The impact of LTβR deficiency on IFNγ pathways is visualized using MetaCore. Lung tissue of uninfected (d0) WT and LTβR$^{-/-}$ mice was used for RNA sequencing. Significant differential gene expression data is superimposed onto the map and visualized as thermometer-like symbols. Upward pointing thermometers are depicted in red and describe upregulated gene expression and downward pointing (blue) thermometers describe downregulated gene expression. Colored lines indicate activation (green), inhibition (red) or unspecified (grey) interactions between the molecules. DEseq2 was used to test for differential expression, "Wald" test and an adjusted p-value of 0.1 were used to determine significance. The object shapes correspond to specific molecule types and all symbols and arrows etc. are described in supplementary figures 2 & 3. The most strongly affected pathways are marked by bold arrows.

Increased translocation of STAT1 to the nucleus could explain the strongly increased IRF8 expression which could also affect IFNγ induced transcription (blue arrows). The enhanced

PLCγ expression could be responsible for the increased expression of ME2b/c, two important transcription factors in muscle, and BAFF, a member of the TNF/TNFR superfamily that is important in lymphocyte proliferation and differentiation (yellow arrows).

Since the LTβR signals via the canonical as well as the alternative NFκB pathway[34] we were interested whether LTβR deficiency might also affect IFNγ signaling via PI3K and NFκB pathways (Fig. 10). On the one hand, increased IFNγ, JAK2 and Fyn but decreased GAB2 gene expression could be detected. However, LTβR deficiency led to elevated PI3K cat class IA expression, which might ultimately modulate translation positively (blue arrows). On the other hand, increased JAK1 and IκB gene expression could be observed possibly explaining the strongly increased expression of IP10, a chemokine and PD-L1, a ligand that blocks T cell activation (black arrows).

LTβR deficiency leads to a decrease of the parasite burden and significantly alters frequencies of T cell subpopulations in brain tissue. We asked whether the enhanced mortality of LTβR$^{-/-}$ mice might be reflected in increased parasite numbers in brain tissue of *T. gondii* infected animals and determined the parasite burden using qRT-PCR. Surprisingly, significantly fewer parasites were detected in brain tissue of *T. gondii* infected LTβR$^{-/-}$ mice compared to WT mice on day 10 post infection (Fig. 11a). Since it is known that IFNγ induces cell autonomous immune responses[43], IFNγ protein expression was analyzed via ELISA (Fig. 11a). Comparable amounts of IFNγ were detected in brain tissue of uninfected (d0) and *T. gondii* infected (d4 – d10 *p.i.*) WT and LTβR$^{-/-}$ mice. We then asked which other factors could be responsible for the decreased parasite burden and analyzed cytokine and T cell surface marker mRNA expression in brain tissue via qRT-PCR (Fig. 11c & d). LTα, LTβ, IL-4 and IL-12p40 mRNA expression stayed comparable between WT and LTβR$^{-/-}$ mice and rose only slightly during *T. gondii* infection in both cohorts, but LTβR$^{-/-}$ mice compared to WT mice exhibited significantly reduced TNF mRNA expression on day 10 post infection. qRT-PCR analysis also revealed significantly decreased mRNA expression of T cell markers (CD3, CD4 and CD8a) in brain tissue of *T. gondii* infected LTβR$^{-/-}$ mice compared to WT mice on days 7 and 10 *p.i.* (Fig. 11d), even though uninfected LTβR$^{-/-}$ (d0 *p.i.*) mice showed significantly increased CD8 T cell mRNA expression compared to WT mice. This indicates an influence of LTβR signaling on the frequencies of T cell subpopulations in the brain of *T. gondii* infected animals.

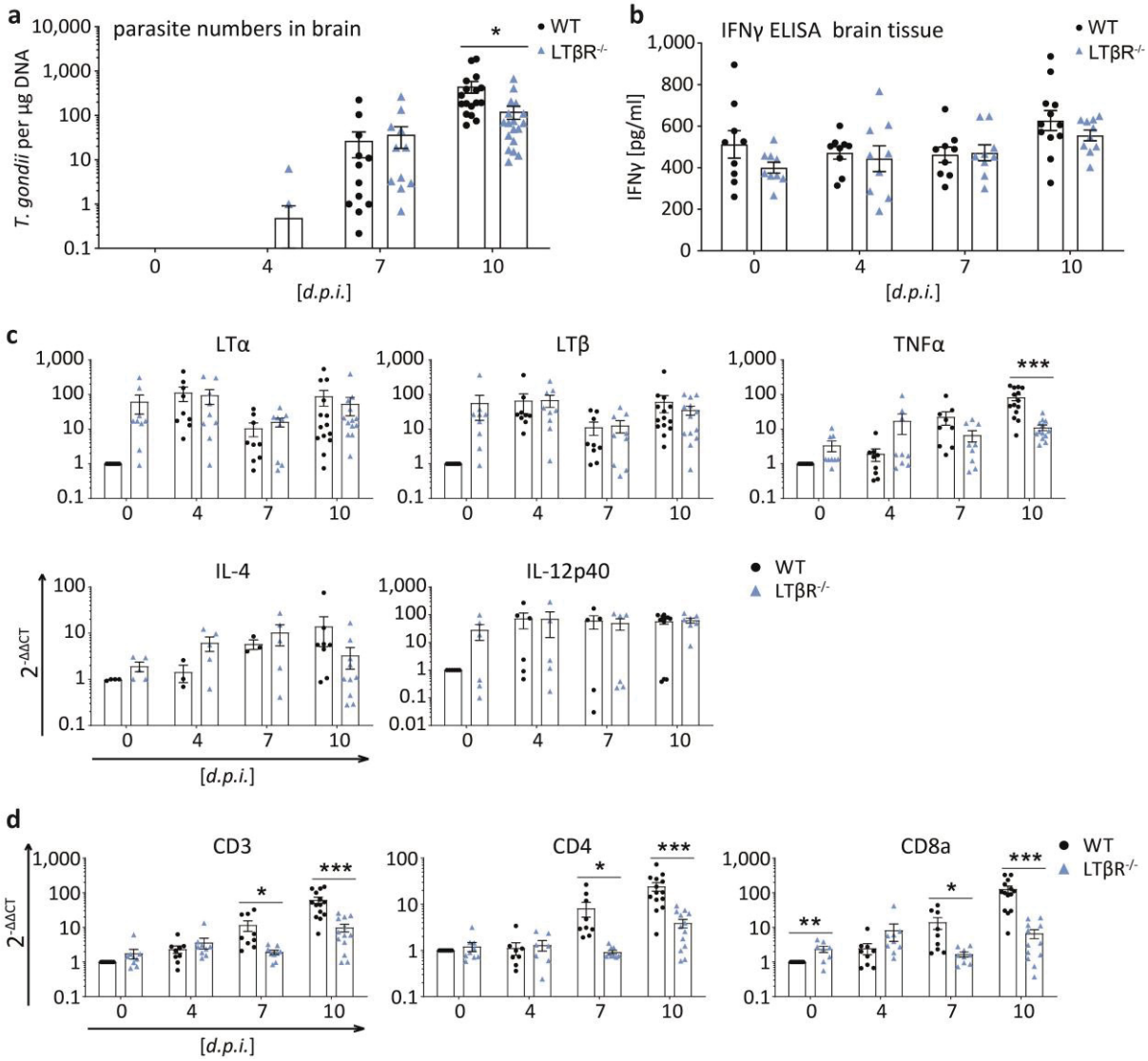

Fig. 11 | Reduced parasite load and less T cell mRNA in brains of LTβR$^{-/-}$ mice. a, qRT-PCR analysis of *T. gondii* DNA in brain tissue of uninfected (d0) and *T. gondii* infected WT and LTβR$^{-/-}$ mice (d0 – 7 *p.i.*: n≥12, d10 *p.i.*: n≥14). **b**, IFNγ ELISA of brain tissue of uninfected and *T. gondii* infected WT and LTβR$^{-/-}$ mice (d0 – 7 *p.i.*: n=9, d10 *p.i.*: n≥10). **c**, mRNA expression of lymphotoxins, TNFα, and interleukins and **d**, T cell markers in brain tissue of uninfected and *T. gondii* infected WT and LTβR$^{-/-}$ mice (d0 – 7 *p.i.*: n=9, d10 *p.i.*: n≥13; exception: IL-4, IL-12p40 d0 – 7 *p.i.*: n≥3, d10 *p.i.*: n≥7) analyzed via qRT-PCR. Data shown are from at least three independent experiments; symbols represent individual animals, bars show mean values ± SEM. Unpaired two-tailed Student's *t*-test was used for statistical analysis. *P<0.05, **P<0.01 and ***P<0.001.

LTβR deficiency leads to decreased expression of IFNγ-regulated molecules in brain tissue. IFNγ-regulated effector molecules are pivotal for containment of *T. gondii*[43], and the important role of IFNγ-induced GTPases, in particular mGBPs[25, 28] and effector molecules such as IDO, iNOS and NOX2 in various tissues is well documented[39, 44, 45, 46]. Although comparable amounts of IFNγ were detected in brain tissue via ELISA (Fig. 11a) mRNA expression of IFNγ-regulated molecules in brain tissue was analyzed via qRT-PCR. Interestingly, while mRNA expression of all mGBPs analyzed increased during the course of infection (d0 – d10 *p.i.*) in

WT animals, LTβR$^{-/-}$ mice showed significantly reduced expression of mGBP1, mGBP2, mGBP3, mGBP8, and mGBP9 already on day 7 post infection and significantly reduced expression for all mGBPs in brain tissue on day 10 post infection (Fig. 12a).

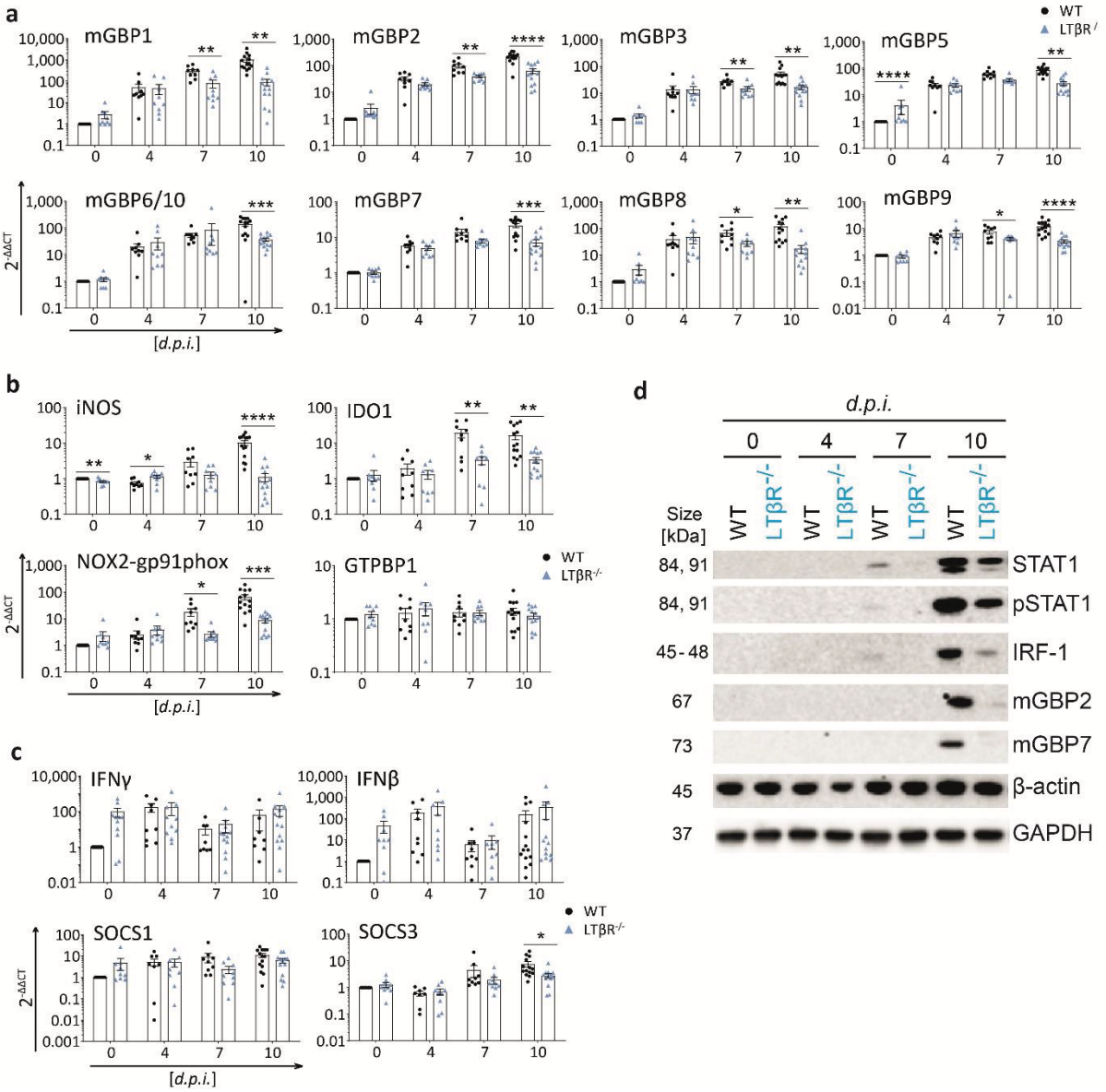

Fig. 12 | Decreased mRNA expression of IFNγ-mediated effector molecules in the brain of LTβR$^{-/-}$ mice. qRT-PCR analysis of **a**, mGBPs, **b**, host effector molecules (iNOS, IDO1, NOX2-gp91phox) as well as an IFNγ unregulated GTPase (GTPBP1) and **c**, interferons, SOCS1 and 3 in brain tissue of uninfected (d0) and *T. gondii* infected WT and LTβR$^{-/-}$ mice (d0 – 7 *p.i.*: n=9, d10 *p.i.*: n≥13; exception: IFNγ d0 – 10 *p.i.* n≥8,). Data shown in **a**, **b**, and **c** are from at least three independent experiments; symbols represent individual animals, bars show mean values ± SEM. Unpaired two-tailed Student's *t*-test was used for statistical analysis. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. **d**, Representative immunoblot analysis of proteins induced via the IFNγ signaling pathway in brain tissue of uninfected and *T. gondii* infected WT and LTβR$^{-/-}$ mice (one experiment, with d0 – 7 *p.i.*: n=3, d10 *p.i.*: n=5).

Surprisingly, mGBP5 is the only mGBP showing increased mRNA expression in brain tissue of uninfected (d0) LTβR$^{-/-}$ mice compared to WT mice. Expression of other effector molecules such as iNOS, IDO1 and NOX2-gp91phox (Fig. 12b) was also significantly reduced in brain tissue of LTβR$^{-/-}$ mice on day 10 post infection. Interestingly, while increased NOX2 mRNA expression was detected in LTβR$^{-/-}$ mice (d0 and d4 *p.i.*), expression did not increase during *T. gondii* infection (d7 and d10 *p.i.*) in contrast to WT animals which showed a significant increase (Fig. 12b). No significant change in expression of the IFNγ-independent GTPase GTPBP1 could be detected in brain tissue of WT or LTβR$^{-/-}$ mice during infection (Fig. 12b). Although decreased mRNA expression of IFNγ-regulated molecules could be detected, IFNγ mRNA expression in brain tissue (Fig. 12c) stayed comparable between WT and LTβR$^{-/-}$ mice throughout *T. gondii* infection. In addition, equivalent mRNA expression of IFNβ as well as SOCS1 could be detected in brain tissue of both genotypes, but for SOCS3, LTβR$^{-/-}$ mice showed decreased mRNA expression on day 10 post infection. Immunoblot assays were performed to analyze protein expression of prototype genes involved in IFNγR signaling as well as IFNγ-regulated genes in brain tissue (Fig. 12d). Levels of STAT1, pSTAT1, IRF-1, mGBP2 and mGBP7 rose in WT mice during the course of infection. LTβR$^{-/-}$ mice showed only minor expression for these proteins in brain tissue on day 10 post infection compared to WT mice.

Establishing a protocol for an immune serum transfer experiment to prolong survival of *T. gondii* infected LTβR$^{-/-}$ mice. Since comparable amounts of IFNγ were detected in lung and brain tissue of LTβR$^{-/-}$ mice we also analyzed IFNγ expression in sera of uninfected (d0) and *T. gondii* infected (d4 – d10 *p.i.*) WT and LTβR$^{-/-}$ mice via ELISA (Fig. 13a). LTβR$^{-/-}$ mice showed continually increasing amounts of IFNγ (d0 – d10 *p.i.*) compared to WT mice which showed already reduced IFNγ on day 10 post infection. Since IFNγ is involved in B cell activation and differentiation we also wanted to investigate the humoral immune response to *T. gondii* infection. Previously, we have shown that T. *gondii* infected LTβR$^{-/-}$ mice cannot mount an IgG response. We therefore decided to establish an immune serum transfer model to assess the immunoglobulin (Ig) M and IgG response to *T. gondii* in LTβR$^{-/-}$ mice and monitor survival in treated vs. untreated mice.

To this end, control serum (from uninfected WT donor mice) and immune serum (from *T. gondii* infected WT donor mice) was collected on day 14 post infection. The presence of IgM and IgG antibodies specific for *T. gondii* proteins in the immune serum was determined using line blots (Fig. 13b) coated with specific recombinant *T. gondii* tachyzoite and bradyzoite antigens (ROP1c, GRA7, GRA8, p30 and MAG1). Different batches of immune serum showed slightly different antibody titers (Fig. 13b). Visual evaluation of the original line blots suggested higher *T. gondii* specific IgG antibody titer in experiment I compared to experiment II. In experiment I,

light band intensities were still visible at a serum dilution of 1:1,600, whereas in experiment II no signal could be visual detected at this dilution. Neither IgM nor IgG antibodies specific for *T. gondii* were detected in control sera but were clearly detectable in immune serum. Two different experimental setups (I & II) were tested to determine the optimal time points for serum transfer (indicated by red arrows) from WT donor mice (control and immune serum) into WT and LTβR$^{-/-}$ acceptor mice to achieve the protection of *T. gondii* infected LTβR$^{-/-}$ mice (Fig. 13c).

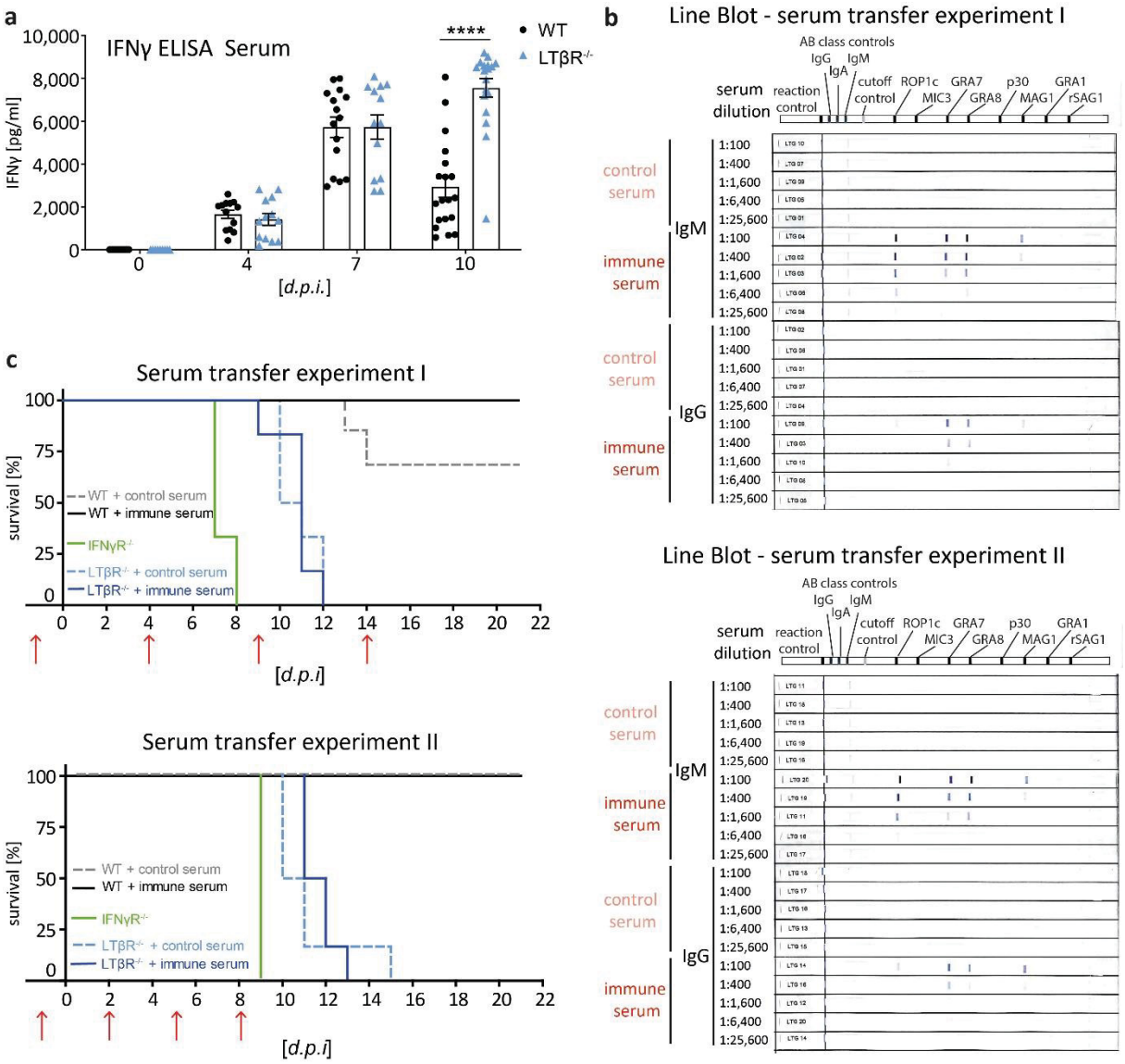

Fig. 13| Increased IFNγ levels in serum of LTβR$^{-/-}$ mice and optimization of serum transfer experiments. a, IFNγ ELISA of serum from uninfected and *T. gondii* infected WT and LTβR$^{-/-}$ mice (d0 – 7 *p.i.*: n≥10, d10 *p.i.*: n≥18). **b**, Detection of IgM and IgG antibodies against *T. gondii* in diluted serum (1:100 – 1:25,600) of uninfected (control serum) and *T. gondii* infected (immune serum ME49, 20 cysts, *i.p.*) donor WT mice. Survival experiments of **c**, serum transfer (indicated by red arrows) from uninfected donor WT mice (control serum) or from donor WT mice infected with *T. gondii* (immune serum) into WT and LTβR$^{-/-}$ acceptor mice. On day 0 acceptor mice (n=6) were infected with *T. gondii* (ME49, 10 cysts, *i.p.*) and survival was evaluated. IFNγR$^{-/-}$ mice (n=3) served as infection controls. Data shown in **b** and **c** show two independent experiments. Data shown in **a** are from four independent experiments; symbols represent individual animals, bars show mean values ± SEM. Unpaired two-tailed Student's *t*-test was used for statistical analysis. ****P<0.0001.

On day 0 acceptor mice were infected with *T. gondii* and survival was evaluated. These experiments demonstrate that an immune serum transfer every five days (one day prior to infection and days 4, 9 and 14 *p.i.*) is less effective compared to immune serum transfer every three days (one day prior to infection and days 2, 5 and 8 *p.i.*), demonstrated by an initially slightly better survival rate of *T. gondii* infected LTβR$^{-/-}$ mice. IFNγR$^{-/-}$ mice served as infection control and succumbed around day 8 or 9 post infection[47], indicating successful *T. gondii* infection.

Discussion

The results obtained in this study have revealed that LTβR deficiency dysregulates the immune responses in multiple organs including spleen, lung and brain tissue. LTβR$^{-/-}$ mice show altered numbers of immune cell populations in the spleen, increased IFNγ production in serum and fewer parasites in the brain. LTβR deficiency did not affect IFNγ protein expression in lung and brain tissue, but altered global gene expression in lung tissue and clearly affected IFNγ mediated gene as well as protein expression in lung and brain tissue.

Since we previously showed that infected WT compared to LTβR$^{-/-}$ mice have increased spleen weight, but reduced splenocyte numbers early in infection (d0 – d7 *p.i.)*[39], we analyzed immune cell populations in infected animals. Our results show increased numbers of immune cells in spleens of WT mice compared to LTβR$^{-/-}$ mice, they therefore cannot be responsible for the reduced splenocyte count in WT animals. Further analysis of splenocytes will be necessary to clarify the cause of the decreased cellularity of the spleens of infected WT animals and increased numbers of splenocytes in infected LTβR$^{-/-}$ animals respectively. Preliminary observations suggest that LTβR$^{-/-}$ mice have significantly increased numbers of apoptotic cells (data not shown). Increased numbers of apoptotic splenocytes are also observed in *T. gondii* infected aryl hydrocarbon receptor deficient mice (personal communication, I. Förster and S. Cengiz, LIMES Institute, University of Bonn).

Flow cytometry data shows significant differences between LTβR$^{-/-}$ and WT mice in the composition of splenic cell populations, which can be explained by the role of LTβR signaling in the generation and homeostasis of the architecture of secondary lymphoid organs and in the differentiation and maturation of immune cell populations[35]. The differences observed during the course of infection, e.g. the absence of increasing DC or macrophages numbers late in infection in LTβR$^{-/-}$ animals is probably due to the dysregulation of IFNγ dependent processes which is discussed in detail below. Interestingly, tSNE analysis shows a slightly different picture: Here, infected and uninfected LTβR$^{-/-}$ and WT animals do not show very obvious differences. Numbers of DCs seem similar, the only marked differences between the

two genotypes at any given time point are the reduced numbers of neutrophils and the almost complete lack of eosinophils in infected LTβR$^{-/-}$ animals. One explanation for these discrepancies is of course the fact that Flow cytometry data depicts absolute cell numbers and tSNE data shows cell frequencies. tSNE analysis is an unbiased machine learning algorithm that integrates the data of many cell markers simultaneously. It can therefore be used to better visualize the subtle shifting of populations. This can be observed for macrophages (Fig. 2b) where in uninfected animals nearly all cells that express the appropriate markers (orange) are on the right hand side, whereas in infected animals approximately half of these cells appear on the left side. This indicates that the repertoire of surface markers has changed, either by expression of additional markers or by a reduction of marker expression. Notably, in LTβR$^{-/-}$ animals the analyzed cell populations show the same shift in cell populations as WT animals, suggesting, that in LTβR$^{-/-}$ animals changes of the surface marker repertoire are mostly unaffected, suggesting unimpaired cell differentiation.
Numbers of naïve CD4^{+} T cells in spleens of LTβR$^{-/-}$ mice did not decrease from day 7 to day 10 post infection as observed in WT mice (Fig. 3a). This decrease of naïve CD4^{+} T cells is expected due to the fact that naïve precursor T cells develop into memory and effector T cell subtypes. It is possible that in LTβR deficient mice naïve T cells are not able to migrate into secondary lymphoid tissues (i.e. lymph nodes) as they do in WT mice where naïve T cells interact with DCs in secondary lymphoid tissues[48].

It is known that IFNγ and IL-6 activate STAT1 and STAT3 which in turn leads to SOCS1 and SOCS3 transcription. Expression of both SOCS1 and SOCS3 then inhibit JAK phosphorylation of STAT proteins thus creating a negative feedback loop[32]. We previously described[39] increased IFNγ mRNA expression in lung tissue of uninfected and infected LTβR$^{-/-}$ mice. In this study, we found increased SOCS1 and SOCS3 mRNA expression in uninfected LTβR$^{-/-}$ mice and, unexpectedly, comparable IFNγ protein expression between WT and LTβR$^{-/-}$ mice during *T. gondii* infection. These somewhat conflicting data suggests on the one hand, that IFNγ expression must be regulated on a post-transcriptional level and on the other hand that SOCS1 and 3 upregulation could, at least in part, be IFNγ independent.

Transcriptomic data was analysed by PCA and a heatmap was created (Fig. 5). This data uncovered a different activation status of genes in uninfected LTβR$^{-/-}$ compared to WT mice. Possibly the lack of LTβR signaling is compensated by excess upregulation of other genes (e.g. TNF) which in turn activates or even necessitates induction of negative feedback loops to avoid or mitigate excessive upregulation of the immune system. This could explain the generally ‘upregulated’ state in the LTβR$^{-/-}$ compared to the WT mice, that was also observed in a volcano plot. Visualization of transcriptomic data as a volcano plot and corresponding

GSEA (Fig. 6) revealed that LTβR deficiency affects Ig gene expression, B cell signaling, and antigen presentation. Preliminary analysis of transcriptome data (Supplementary Fig. 1) reports downregulated IFNγ responses and reduced expression of histones in infected (d7 *p.i.*) compared to uninfected LTβR$^{-/-}$ animals. This could influence chromatin assembly and nucleosome organization as well as post-translational modifications of histones such as acetylation, methylation, phosphorylation and ubiquitination[49, 50]. These post-translational modifications play important roles in gene regulation, DNA repair as well as mitosis and meiosis[51]. This could also be one cause of the generally different activation status of uninfected LTβR$^{-/-}$ mice and might contribute to a less efficient immune response to *T. gondii* infection. We hypothesize that while LTβR$^{-/-}$ mice do not show an overt phenotype in controlled housing conditions (specified pathogen-free) and are able to compensate for the lack of LTβR mediated signaling, this is a precarious balance that is profoundly disturbed when the animals are challenged with a pathogen.

Previously, we have shown that uninfected LTβR$^{-/-}$ mice show an increased expression of cytokines[39] and confirm in this study that these animals possess an increased genes expression pattern (Fig. 5a & 6a) compared to WT mice, suggesting a higher activation status of these animals in homeostasis. In contrast, after infection LTβR$^{-/-}$ mice show an overall diminished upregulation of gene expression (Fig. 5a) compared to WT animals. These data are consistent with the hypothesis that in *T. gondii* infection the upregulation of the immunologic processes initiated to contain the parasite results in a higher activation of the immune response, further dysregulating this precarious balance and resulting in a paralysis of the whole system. It could also explain not only why uninfected WT and LTβR$^{-/-}$ mice are so dissimilar in the PCA (Fig. 5b) but also why their infected counterparts show a markedly different gene expression profile. It has also to be kept in mind that these data show the responsiveness of infected LTβR$^{-/-}$ mice on day 7 post infection, experimental data paints an even more obvious picture on day 10 post infection. To summarize, RNAseq analysis revealed fundamental differences in gene expression patterns of uninfected and *T. gondii* infected LTβR$^{-/-}$ mice compared to uninfected and infected WT mice.

In an attempt to assess the RNAseq data in a more global context MetaCore pathway analysis was performed to investigate the possible interactions between LTβR and IFNγ signaling. Notably, the lack of LTβR signaling seems to slightly increase IFNγ expression that could activate and increase downstream signaling. IFNγ is a well-described key regulator of many pathways involved in innate and adaptive immunity. IFNγ production is upregulated in most viral, bacterial and parasitic infections to initiate an immune response. A slight increase in IFNγ signaling in LTβR$^{-/-}$ mice could - even in the absence of pathogens - induce a lowgrade proinflammatory status[52]. Increased TNF and IP10 expression (induced via JAK/STAT and

MAPK pathways), increased CD40 and CCL2 expression (via STAT1) and an increased type I interferon response expression could all contribute towards this inflammatory status, without actually leading to a manifest phenotype in unchallenged LTβR$^{-/-}$ mice. In addition, STAT1 mediated signaling could be responsible - via IRF8 – for increased expression of gp91-phox and p67-phox both involved in the production of reactive oxygen species (ROS)[53], ultimately also contributing to the proinflammatory status. On the other hand, ROS can reinforce pro-apoptotic signaling, which may already be enhanced by STAT3 and NFκB mediated signals. These interactions could explain the shift in the various immune cell lineages seen in LTβR$^{-/-}$ mice. Moreover, this pathway analysis reveals a so far undescribed specific interaction between STAT1, IRF8 and mGBP4. However, since it has been reported that the mGbp4 gene is not translated into a functional protein in C57BL/6 mice, this connection is probably irrelevant[54]. BAFF upregulation via PLCγ which would influence lymphocyte and especially B cell activation and differentiation could explain the major dysregulation of the humoral immune response observed in *T. gondii* infected mice. For all of that, it has to be kept in mind that a pathway analysis correlates experimental mRNA expression data with published protein interactions. Regulation of translational processes and post-translational modifications are not taken into account. While the MetaCore pathway analysis clearly demonstrates that LTβR deficiency could affect IFNγ signaling via multiple pathways it cannot answer the question of how LTβR signaling interacts with IFNγ signaling on a molecular level.

Increased susceptibility of LTβR$^{-/-}$ mice to *T. gondii* infection and increased parasite numbers in lung, spleen und muscle tissue have been reported[28, 39]. The trigger for the migration of infectious *T. gondii* tachyzoites into the brain is not completely understood. However, it is clear that under pressure of the host immune response, tachyzoites convert to metabolically less active bradyzoites. These reside in tissue cysts, preferentially in the brain and musculature, where they are able to evade the immune system establishing chronic toxoplasmosis[4]. The significantly lower parasite numbers detected in the brain of LTβR$^{-/-}$ mice on day 10 post infection (Fig. 11 a) could be due to the lack of specific cell populations (e.g. DCs or macrophages) that are used by the parasite as a “Trojan horse” to shuttle into the brain[55, 56, 57]. Reduced numbers of DCs in LTβR$^{-/-}$ mice were reported[58]. In alignment with this, the number of DCs (d4 *p.i.*), especially cDCs and pDCs (d0 – d10 *p.i.*) were significantly reduced in spleens of LTβR$^{-/-}$ mice (Fig. 1b & Fig. 2) suggesting that due to fewer DCs fewer parasites are being shuttled to the brain. This “Trojan horse” hypothesis is not limited to *T. gondii*. *Leishmania major*, another intracellular parasite, is described to hijack neutrophil granulocytes and inflammatory DCs to invade its hosts macrophages, where it survives and multiplies resulting in disease progression[59, 60, 61]. On the other hand, lower parasite numbers could (also) be due to the lack of timely immune pressure to force the parasite into the chronic stage of infection.

In addition, LTβR$^{-/-}$ mice demonstrated reduced expression of CD4 and CD8 mRNA expression in brain tissue. This implies that T cell mediated immune responses are probably not responsible for the lower parasite burden in the brain. So far, a protective role of CD4^{+} T cells in *T. gondii* infection could be shown only in splenocytes[62], but the role of CD4^{+} T cells in the brain of *T. gondii* infected mice is largely unexplored. On the other hand, it has been shown that perforin-mediated CD8^{+} T cell activity can eliminate *T. gondii* cysts from the brain in an IFNγ independent manner[63]. In this context it is interesting to note that IFNγ levels in the brain of WT animals do not increase during infection (Fig. 11b).

IFNγ regulated expression of molecules important for cellular effector functions such as iNOS, IDO1 and NOX2, as well as the upregulation of all mGBPs which are essential for an efficient immune response to *T.gondii*[23, 24, 64, 65] is significantly reduced in brain tissue of LTβR$^{-/-}$ animals (Fig. 12). Interestingly, the brain tissue of WT mice did not show increased IFNγ expression on the mRNA or protein level during infection. Therefore, the increased expression of anti-parasitic effector molecules observed in brain tissue of infected WT mice must be independent of IFNγ upregulation. The lack of upregulation of anti-parasitic effector molecules in infected LTβR$^{-/-}$ animals points to additional signaling defects in these animals. The role of IFNγ induced effector molecules such as mGBPs in the brain of *T. gondii* infected mice is largely unknown especially in terms of conversion to and maintenance of the chronic phase of infection. Increased parasite numbers, reduced mRNA and protein expression of IFNγ-regulated effector molecules as well as reduced IFNγ in serum early in infection in lung tissue of *T. gondii* infected (d0 – d10 *p.i.*) LTβR$^{-/-}$ mice were previously reported[39]. These mice also lack a significant NK cell population, leading to the hypothesis that the delayed IFNγ response in LTβR$^{-/-}$ mice is at least in part caused by the absence of IFNγ production by NK cells[39] which is one of the initial and essential steps in the immune response to *T. gondii*[66, 67].

LTβR$^{-/-}$ animals do not mount an IgG response against *T. gondii*[39]. Initial experiments (Fig. 13b and c) were performed to determine the optimal experimental parameters to achieve prolonged survival. The fact that an earlier start of transferring immune serum and shorter intervals of serum transfer only resulted in slightly improved survival rates of acceptor LTβR$^{-/-}$ mice is most probably due to the quality of the immune serum: This assumption is based on the observation that donor animals used for the collection of immune serum for experiment II showed signs of a more severe acute *T. gondii* infection than donor mice used for experiment I, leading us to assume that their immune response was not as efficient. While exact quantification of relative signal intensities of parasite specific antibodies was not performed, relative antibody titers were visually assessed, suggesting higher antibody titers in experiment I compared to experiment II. One out of six acceptor LTβR$^{-/-}$ animals that received control serum (Fig. 13c, experiments II) survived until day 15 post infection. We believe that this mouse is an outlier, most probably

due to a technical error (e.g. was possibly injected with fewer cysts), because we have never observed a *T. gondii* infected LTβR$^{-/-}$ animal surviving until that time point. This underscores the need for further refining this promising *in vivo* infection model.

The data shows that LTβR deficiency dysregulates immune cell populations in the spleen, concomitantly increases IFNγ production in serum but decreases parasite numbers in the brain. It does not affect IFNγ protein expression in lung and brain tissue, but alters global gene expression in lung tissue and clearly affects gene as well as protein expression involved in IFNγ signaling in lung and brain tissue. In addition, a serum transfer model for *T. gondii* infection was established. This work highlights the complex role of the LTβR in *T. gondii* infection and identifies multiple starting points for further research that is necessary to gain new insights into the pathology of *T. gondii,* which could provide new therapeutic strategies for the treatment of human toxoplasmosis.

Methods

Mice. LTβR$^{-/-}$ mice were previously described[30] and have been backcrossed for at least 10 generations onto a C57BL/6 background. Wild-type (WT) littermates were used as controls. Mice were kept under specific pathogen-free conditions (SPF) in the animal facility at the Heinrich Heine University Düsseldorf and were 8-16 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# 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 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 post infection (*d.p.i*), uninfected mice served as controls. After euthanasia (100 mg/kg Ketamin, 10 mg/kg Xylazin, Vétoquinol GmbH) blood was taken from the Vena cava inferior and spleen, brain and lung tissue was harvested for analysis.

Detection of parasite load. Total DNA was isolated from brain tissue using a DNA isolation kit (Genekam) according to the manufacturer's protocol. qRT-PCR was performed on the Bio-Rad CFX-96 Touch-Real-Time Detection System. TgB1 primers and probe (Metabion) were used to amplify a defined section of the 35-fold repetitive B1 gene from *T. gondii*

(Supplementary Table 1). A *T. gondii* DNA standard curve was generated and used to determine B1 amplification for calculation of parasite load.

Real-time qRT-PCR. Total RNA was isolated from tissues via the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was reversely transcribed using M-MLV reverse transcriptase (Invitrogen). qRT-PCR was performed on the Bio-Rad CFX-96 Touch-Real-Time Detection System. Primer (Metabion) sequences and corresponding probes (Metabion, Roche & TipMolBIOL) are listed in Supplementary Table 1. Results are expressed relative to expression in untreated WT mice normalized to β-*actin* ($2^{-\Delta\Delta CT}$).

ELISA. Serum as well as protein preparations isolated from brain and lung tissue were analyzed for IFNγ using DuoELISA (DY485, R&D Systems) according to the manufacturer's protocol.

RNAseq analysis. Lung tissue of uninfected (d0) und *T. gondii* infected (ME49 strain, 40 cysts*, i.p.*) WT and LTβR$^{-/-}$ mice was obtained and RNA sequencing was performed on a HiSeq3000 device. Mouse and *T. gondii* transcripts were quantified from fastq files using Salmon with default settings and GCbias 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 hypothetical genes or from pseudogenes or with retained introns were excluded prior to conversion to gene counts by 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 + Genotype: Infection. For determining significance the Wald test with an adjusted p-value of 0.1 was used.

Gene Set Enrichment Analysis. For GSEA, GO (Biological Process) molecular signatures were obtained using the msigdr package in R. Gene-level stats for each differential expression contrast were tested for geneset enrichment using the fgsea package, with an adjusted p-value of 0.1.

Pathway analysis. The web-based bioinformatics suite MetaCore (Clarivate Analytics; https://portal.genego.com) was used to perform integrated pathway and network analysis. Differential gene expression data of RNAseq analysis from uninfected (d0) WT and LTβR$^{-/-}$ mice were used as input and illustrated as pathway maps. DEseq2 was used to test for differential expression, the Wald test with an adjusted p-value of 0.1 was used for determining significance.

Immunoblot analysis and antibodies. Brain tissue was homogenized in PBS containing cOmplete^{TM} Protease Inhibitor Cocktail (Roche) using the Precellys® homogenizer (Bertin).

Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific™) according to the manufacturer's protocol. Samples were separated by 4-12% SDS-PAGE, followed by electrophoretic transfer to nitrocellulose membranes before blocking and incubation with primary antibodies listed in Supplementary Table 2. HRP-labeled anti-rabbit or anti-mouse antibodies (Cell Signaling Technologies) were used as secondary antibodies.

Flow cytometry. Single-cell suspended splenocytes were stained with the Fixable Viability Dye eFluor® 780 (eBioscience™). Surface staining with antibodies specific for MHC II (M5/114.15.2), CD11c (N418), CD11b (M1/70), BST2 (927), F4/80 (BM8), Ly6C (RB6-8C5) all purchased from BioLegend, was performed. For intracellular staining, splenocytes were incubated for 20 h with toxoplasma lysate antigen [TLA, 15 µg/ml] before adding brefeldin A (eBioscience™) for an additional 4 hours. After surface staining with anti-CD4 (RM4-5), anti-CD8a (53-6.7), anti-CD44 (IM7), and anti-CD62L (MEL-14) cells were fixed, permeabilized and stained with anti-IL-2 (JES6-5H4), anti-IL-4 (11B11), and anti-TNFα (MP6-XT22) (all purchased from BioLegend) using Fix & Perm® Cell Permeabilization Kit (Life Technologies) according to the manufacturer's protocol. BD Calibrate beads (BD Bioscience) were added to the samples before acquisition with a BD LSRFortessa.

tSNE. The cloud-based platform Cytobank®[68] (Mountain View) was used for visualization of flow cytometry data. Analysis was performed on pregated non T and non B cells using 27,000 events per sample (parameters: iterations 1,008, perplexity 80, Theta 0.5) before overlaid dot plots were generated.

Detection of *T. gondii* specific antibodies. *Recom*Line *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 IgG-HRP-labeled (Invitrogen) conjugates. Otherwise, the assay was performed according to the manufacturer's protocol.

Serum transfer. Serum from naïve donor mice (control serum) or WT mice infected with 20 cysts of the ME49 strain of *T. gondii* (immune serum) was collected, and 0.2 ml of serum was transferred intraperitoneally to acceptor WT and LTβR$^{-/-}$ mice one day prior to infection (d-1) as well as on days 4, 9 and 14 *p.i.* (serum transfer experiment I) or on days -1, 2, 5 and 8 *p.i.* (serum transfer experiment II), respectively. Acceptor (WT and LTβR$^{-/-}$ mice) as well as IFNγ$^{-/-}$ 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 (Version8, GraphPad) using two-tailed Student's *t*-test. Symbols represent individual animals, bars show mean values ± SEM. P values of ≤0.05 were considered statistically significant and marked with asterisks. P values of ≥0.05 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.

References

1. Flegr J, Prandota J, Sovickova M, Israili ZH. Toxoplasmosis--a global threat. Correlation of latent toxoplasmosis with specific disease burden in a set of 88 countries. *PLoS One* 2014, **9**(3): e90203.

2. Montoya JG, Liesenfeld O. Toxoplasmosis. *Lancet* 2004, **363**(9425): 1965-1976.

3. Hartmann A. Multiple routes of phosphatidylethanolamine biogenesis ensure membrane integrity of Toxoplasma gondii. Humboldt University Berlin, 2016.

4. Lyons RE, McLeod R, Roberts CW. Toxoplasma gondii tachyzoite-bradyzoite interconversion. *Trends in parasitology* 2002, **18**(5): 198-201.

5. Gaddi PJ, Yap GS. Cytokine regulation of immunopathology in toxoplasmosis. *Immunol Cell Biol* 2007, **85**(2): 155-159.

6. Scharton-Kersten T, Nakajima H, Yap G, Sher A, Leonard WJ. 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* 1998, **160**(6): 2565-2569.

7. Denkers EY. T lymphocyte-dependent effector mechanisms of immunity to Toxoplasma gondii. *Microbes Infect* 1999, **1**(9): 699-708.

8. Vremec D, O'Keeffe M, Hochrein H, Fuchsberger M, Caminschi I, Lahoud M, et al. Production of interferons by dendritic cells, plasmacytoid cells, natural killer cells, and interferon-producing killer dendritic cells. *Blood* 2007, **109**(3): 1165-1173.

9. Kraaij MD, Vereyken EJ, Leenen PJ, van den Bosch TP, Rezaee F, Betjes MG, et al. Human monocytes produce interferon-gamma upon stimulation with LPS. *Cytokine* 2014, **67**(1): 7-12.

10. Darwich L, Coma G, Pena R, Bellido R, Blanco EJ, Este JA, et al. Secretion of interferon-gamma by human macrophages demonstrated at the single-cell level after costimulation with interleukin (IL)-12 plus IL-18. *Immunology* 2009, **126**(3): 386-393.

11. Suzuki Y, Orellana MA, Schreiber RD, Remington JS. Interferon-gamma: the major mediator of resistance against Toxoplasma gondii. *Science* 1988, **240**(4851): 516-518.

12. Yap GS, Sher A. Cell-mediated immunity to Toxoplasma gondii: initiation, regulation and effector function. *Immunobiology* 1999, **201**(2): 240-247.

13. Saeij JP, Frickel EM. Exposing Toxoplasma gondii hiding inside the vacuole: a role for GBPs, autophagy and host cell death. *Curr Opin Microbiol* 2017, **40**: 72-80.

14. Daubener W, Remscheid C, Nockemann S, Pilz K, Seghrouchni S, Mackenzie C, et al. Anti-parasitic effector mechanisms in human brain tumor cells: role of interferon-gamma and tumor necrosis factor-alpha. *Eur J Immunol* 1996, **26**(2): 487-492.

15. Fox BA, Gigley JP, Bzik DJ. Toxoplasma gondii lacks the enzymes required for de novo arginine biosynthesis and arginine starvation triggers cyst formation. *Int J Parasitol* 2004, **34**(3): 323-331.

16. Pfefferkorn ER, Rebhun S, Eckel M. Characterization of an indoleamine 2,3-dioxygenase induced by gamma-interferon in cultured human fibroblasts. *J Interferon Res* 1986, **6**(3): 267-279.

17. Murray HW, Szuro-Sudol A, Wellner D, Oca MJ, Granger AM, Libby DM, et al. Role of tryptophan degradation in respiratory burst-independent antimicrobial activity of gamma interferon-stimulated human macrophages. *Infect Immun* 1989, **57**(3): 845-849.

18. Scharton-Kersten TM, Yap G, Magram J, Sher A. Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen Toxoplasma gondii. *J Exp Med* 1997, **185**(7): 1261-1273.

19. Khan IA, Matsuura T, Kasper LH. Inducible nitric oxide synthase is not required for long-term vaccine-based immunity against Toxoplasma gondii. *J Immunol* 1998, **161**(6): 2994-3000.

20. Chang HR, Pechere JC. Macrophage oxidative metabolism and intracellular Toxoplasma gondii. *Microbial pathogenesis* 1989, **7**(1): 37-44.

21. Murray HW, Juangbhanich CW, Nathan CF, Cohn ZA. Macrophage oxygen-dependent antimicrobial activity. II. The role of oxygen intermediates. *J Exp Med* 1979, **150**(4): 950-964.

22. Degrandi D, Konermann C, Beuter-Gunia C, Kresse A, Wurthner J, Kurig S, et al. Extensive characterization of IFN-induced GTPases mGBP1 to mGBP10 involved in host defense. *J Immunol* 2007, **179**(11): 7729-7740.

23. Steffens N, Beuter-Gunia C, Kravets E, Reich A, Legewie L, Pfeffer K, et al. Essential Role of mGBP7 for Survival of Toxoplasma gondii Infection. *mBio* 2020, **11**(1).

24. Yamamoto M, Okuyama M, Ma JS, Kimura T, Kamiyama N, Saiga H, et al. A cluster of interferon-gamma-inducible p65 GTPases plays a critical role in host defense against Toxoplasma gondii. *Immunity* 2012, **37**(2): 302-313.

25. Kutsch S, Degrandi D, Pfeffer K. Immediate lymphotoxin beta receptor-mediated transcriptional response in host defense against L. monocytogenes. *Immunobiology* 2008, **213**(3-4)**:** 353-366.

26. Finethy R, Jorgensen I, Haldar AK, de Zoete MR, Strowig T, Flavell RA, et al. Guanylate binding proteins enable rapid activation of canonical and noncanonical inflammasomes in Chlamydia-infected macrophages. *Infect Immun* 2015, **83**(12): 4740-4749.

27. Wandel MP, Pathe C, Werner EI, Ellison CJ, Boyle KB, von der Malsburg A, et al. GBPs Inhibit Motility of Shigella flexneri but Are Targeted for Degradation by the Bacterial Ubiquitin Ligase IpaH9.8. *Cell Host Microbe* 2017, **22**(4): 507-518 e505.

28. Behnke K, Sorg UR, Gabbert HE, Pfeffer K. The Lymphotoxin beta Receptor Is Essential for Upregulation of IFN-Induced Guanylate-Binding Proteins and Survival after Toxoplasma gondii Infection. *Mediators Inflamm* 2017, **2017**: 7375818.

29. Deckert-Schluter M, Bluethmann H, Rang A, Hof H, Schluter D. Crucial role of TNF receptor type 1 (p55), but not of TNF receptor type 2 (p75), in murine toxoplasmosis. *J Immunol* 1998, **160**(7)**:** 3427-3436.

30. Yap GS, Scharton-Kersten T, Charest H, Sher A. Decreased resistance of TNF receptor p55- and p75-deficient mice to chronic toxoplasmosis despite normal activation of inducible nitric oxide synthase in vivo. *J Immunol* 1998, **160**(3)**:** 1340-1345.

31. Costa FR, Mota CM, Santiago FM, Silva MV, Ferreira MD, Fonseca DM*, et al.* GITR Activation Positively Regulates Immune Responses against Toxoplasma gondii. *PLoS One* 2016, **11**(3)**:** e0152622.

32. Ehlers S, Holscher C, Scheu S, Tertilt C, Hehlgans T, Suwinski J, et al. The lymphotoxin beta receptor is critically involved in controlling infections with the intracellular pathogens Mycobacterium tuberculosis and Listeria monocytogenes. *J Immunol* 2003, **170**(10): 5210-5218.

33. Spahn TW, Maaser C, Eckmann L, Heidemann J, Lugering A, Newberry R, et al. The lymphotoxin-beta receptor is critical for control of murine Citrobacter rodentium-induced colitis. *Gastroenterology* 2004, **127**(5): 1463-1473.

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

35. Futterer A, Mink K, Luz A, Kosco-Vilbois MH, Pfeffer K. The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity* 1998, **9**(1): 59-70.

36. Wu Q, Sun Y, Wang J, Lin X, Wang Y, Pegg LE, et al. Signal via lymphotoxin-beta on bone marrow stromal cells is required for an early checkpoint of NK cell development. *J Immunol* 2001, **166**(3): 1684-1689.

37. Banks TA, Rickert S, Ware CF. Restoring immune defenses via lymphotoxin signaling: lessons from cytomegalovirus. *Immunol Res* 2006, **34**(3): 243-254.

38. Jin L, Guo X, Shen C, Hao X, Sun P, Li P, et al. Salivary factor LTRIN from Aedes aegypti facilitates the transmission of Zika virus by interfering with the lymphotoxin-beta receptor. *Nat Immunol* 2018, **19**(4): 342-353.

39. Anne Tersteegen, Ursula R. Sorg , Richard Virgen-Slane, Patrick Petzsch, Karl Köhrer, Daniel Degrandi, Carl F. Ware, Klaus Pfeffer. Lymphotoxin β receptor: A crucial role in innate and adaptive immune responses against Toxoplasma gondii. Manuscript submitted; 2020.

40. Weiss LM, Dubey JP. Toxoplasmosis: A history of clinical observations. *Int J Parasitol* 2009, **39**(8): 895-901.

41. Durham GA, Williams JJL, Nasim MT, Palmer TM. Targeting SOCS Proteins to Control JAK-STAT Signalling in Disease. *Trends in pharmacological sciences* 2019, **40**(5): 298-308.

42. Wormald S, Zhang JG, Krebs DL, Mielke LA, Silver J, Alexander WS, et al. The comparative roles of suppressor of cytokine signaling-1 and -3 in the inhibition and desensitization of cytokine signaling. *The Journal of biological chemistry* 2006, **281**(16): 11135-11143.

43. Yarovinsky F. Innate immunity to Toxoplasma gondii infection. *Nat Rev Immunol* 2014, **14**(2): 109-121.

44. Ratna A, Arora SK. Leishmania recombinant antigen modulates macrophage effector function facilitating early clearance of intracellular parasites. *Trans R Soc Trop Med Hyg* 2016, **110**(10): 610-619.

45. Koo SJ, Chowdhury IH, Szczesny B, Wan X, Garg NJ. Macrophages Promote Oxidative Metabolism To Drive Nitric Oxide Generation in Response to Trypanosoma cruzi. *Infect Immun* 2016, **84**(12): 3527-3541.

46. Ufermann CM, Domrose A, Babel T, Tersteegen A, Cengiz SC, Eller SK, et al. Indoleamine 2,3-Dioxygenase Activity During Acute Toxoplasmosis and the Suppressed T Cell Proliferation in Mice. *Front Cell Infect Microbiol* 2019, **9**: 184.

47. Deckert-Schluter M, Rang A, Weiner D, Huang S, Wiestler OD, Hof H, et al. Interferon-gamma receptor-deficiency renders mice highly susceptible to toxoplasmosis by decreased macrophage activation. *Lab Invest* 1996, **75**(6): 827-841.

48. Kabashima K, Banks TA, Ansel KM, Lu TT, Ware CF, Cyster JG. Intrinsic lymphotoxin-beta receptor requirement for homeostasis of lymphoid tissue dendritic cells. *Immunity* 2005, **22**(4): 439-450.

49. Robert R. Rich WTS, Anthony J.Frew, Thomas A. Fleisner, Harry W. Schroeder, Cornelia M. Weyand. Clinical Immunology: Principles & practice, vol. 5th edition. Elsevier, 2009.

46. Mersfelder EL, Parthun MR. The tale beyond the tail: histone core domain modifications and the regulation of chromatin structure. *Nucleic acids research* 2006, **34**(9): 2653-2662.

50. Tropberger P, Schneider R. Scratching the (lateral) surface of chromatin regulation by histone modifications. *Nature structural & molecular biology* 2013, **20**(6): 657-661.

51. Stillman B. Histone Modifications: Insights into Their Influence on Gene Expression. *Cell* 2018, **175**(1): 6-9.

52. Codrici E, Albulescu L, Popescu ID, Mihai S, Enciu AM, Albulescu R, et al. Caveolin-1-Knockout Mouse as a Model of Inflammatory Diseases. *Journal of immunology research* 2018, **2018**: 2498576.

53. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive oxygen species in inflammation and tissue injury. *Antioxidants & redox signaling* 2014, **20**(7): 1126-1167.

54. Konermann C, Kresse A, Beuter-Gunia C, Wurthner J, Degrandi D, Pfeffer K, et al. In silico and in vitro characterization of mGBP4 splice variants. *DNA and cell biology* 2007, **26**(12): 847-851.

55. Lambert H, Hitziger N, Dellacasa I, Svensson M, Barragan A. Induction of dendritic cell migration upon Toxoplasma gondii infection potentiates parasite dissemination. *Cellular microbiology* 2006, **8**(10): 1611-1623.

56. Courret N, Darche S, Sonigo P, Milon G, Buzoni-Gatel D, Tardieux I. CD11c- and CD11b-expressing mouse leukocytes transport single Toxoplasma gondii tachyzoites to the brain. *Blood* 2006, **107**(1): 309-316.

57. Lambert H, Vutova PP, Adams WC, Lore K, Barragan A. The Toxoplasma gondii-shuttling function of dendritic cells is linked to the parasite genotype. *Infect Immun* 2009, **77**(4): 1679-1688.

58. Wang YG, Kim KD, Wang J, Yu P, Fu YX. Stimulating lymphotoxin beta receptor on the dendritic cells is critical for their homeostasis and expansion. *J Immunol* 2005, **175**(10)**:** 6997-7002.

59. Laskay T, van Zandbergen G, Solbach W. Neutrophil granulocytes--Trojan horses for Leishmania major and other intracellular microbes? *Trends in microbiology* 2003, **11**(5)**:** 210-214.

60. Regli IB, Passelli K, Hurrell BP, Tacchini-Cottier F. Survival Mechanisms Used by Some Leishmania Species to Escape Neutrophil Killing. *Front Immunol* 2017, **8:** 1558.

61. Hurdayal R, Nieuwenhuizen NE, Khutlang R, Brombacher F. Inflammatory Dendritic Cells, Regulated by IL-4 Receptor Alpha Signaling, Control Replication, and Dissemination of Leishmania major in Mice. *Front Cell Infect Microbiol* 2019, **9:** 479.

62. Grover HS, Blanchard N, Gonzalez F, Chan S, Robey EA, Shastri N. The Toxoplasma gondii peptide AS15 elicits CD4 T cells that can control parasite burden. *Infect Immun* 2012, **80**(9): 3279-3288.

63. Suzuki Y, Wang X, Jortner BS, Payne L, Ni Y, Michie SA, et al. Removal of Toxoplasma gondii cysts from the brain by perforin-mediated activity of CD8+ T cells. *The American journal of pathology* 2010, **176**(4): 1607-1613.

64. Degrandi D, Kravets E, Konermann C, Beuter-Gunia C, Klumpers V, Lahme S, et al. Murine guanylate binding protein 2 (mGBP2) controls Toxoplasma gondii replication. *Proc Natl Acad Sci U S A* 2013, **110**(1): 294-299.

65. Selleck EM, Fentress SJ, Beatty WL, Degrandi D, Pfeffer K, Virgin HWt, et al. Guanylate-binding protein 1 (Gbp1) contributes to cell-autonomous immunity against Toxoplasma gondii. *PLoS Pathog* 2013, **9**(4): e1003320.

66. Denkers EY, Gazzinelli RT, Martin D, Sher A. Emergence of NK1.1+ cells as effectors of IFN-gamma dependent immunity to Toxoplasma gondii in MHC class I-deficient mice. *J Exp Med* 1993, **178**(5): 1465-1472.

67. Gigley JP. The Diverse Role of NK Cells in Immunity to Toxoplasma gondii Infection. *PLoS Pathog* 2016, **12**(2): e1005396.

68. Chen TJ, Kotecha N. Cytobank: providing an analytics platform for community cytometry data analysis and collaboration. *Current topics in microbiology and immunology* 2014, **377**: 127-157.

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Author contributions

A.T. performed and analyzed all experiments, exceptions for Fig. 5 – 10 and Supplementary Fig. 1 (analyzed and visualized by R.V.S). P.P. and K.K. performed RNA sequencing. A.T., U.R.S. and K.P. wrote the manuscript with input from D.D and C.F.W; K.P., U.R.S. and A.T. designed the study.

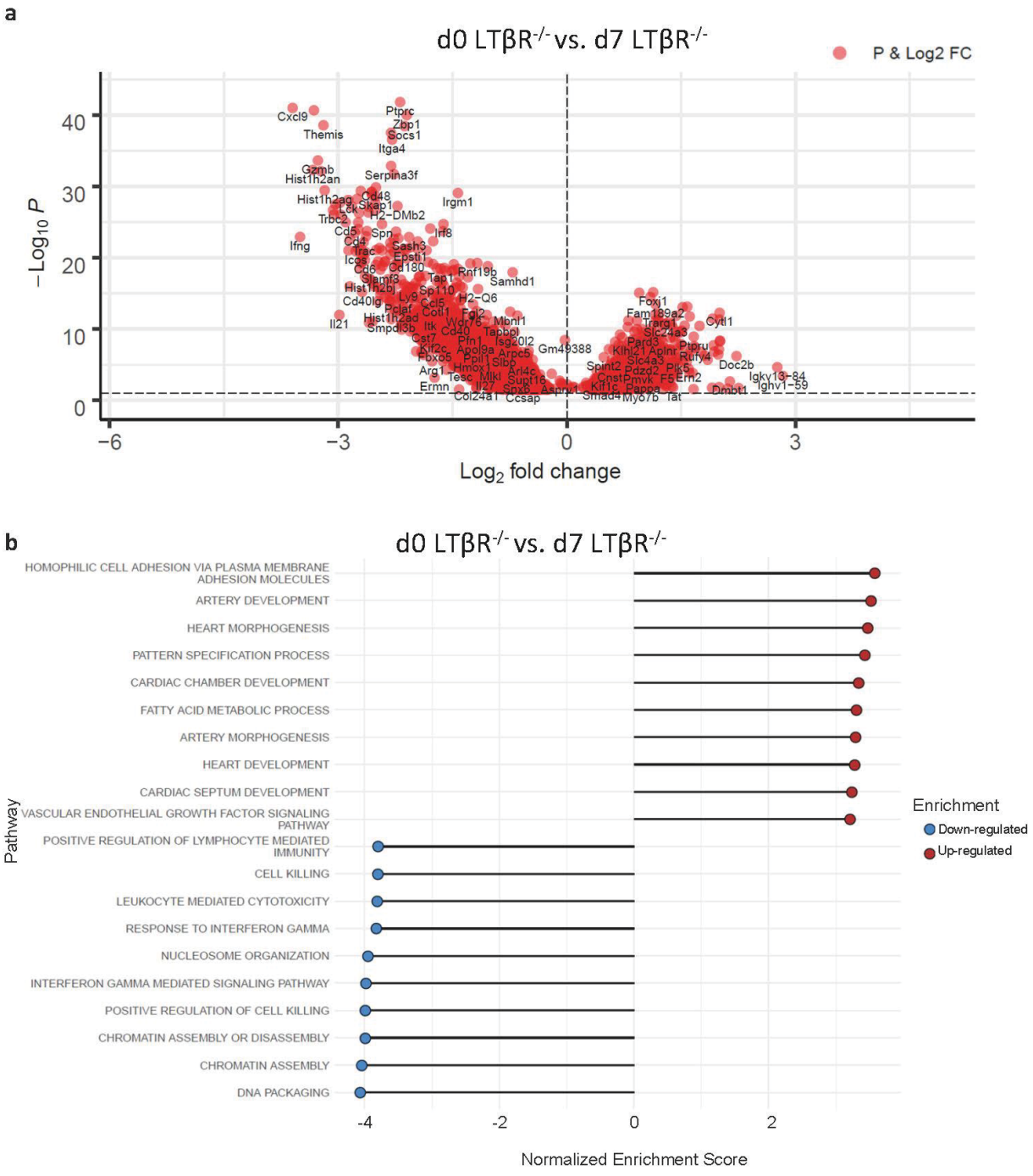
Competing interests

The authors declare no competing interests.

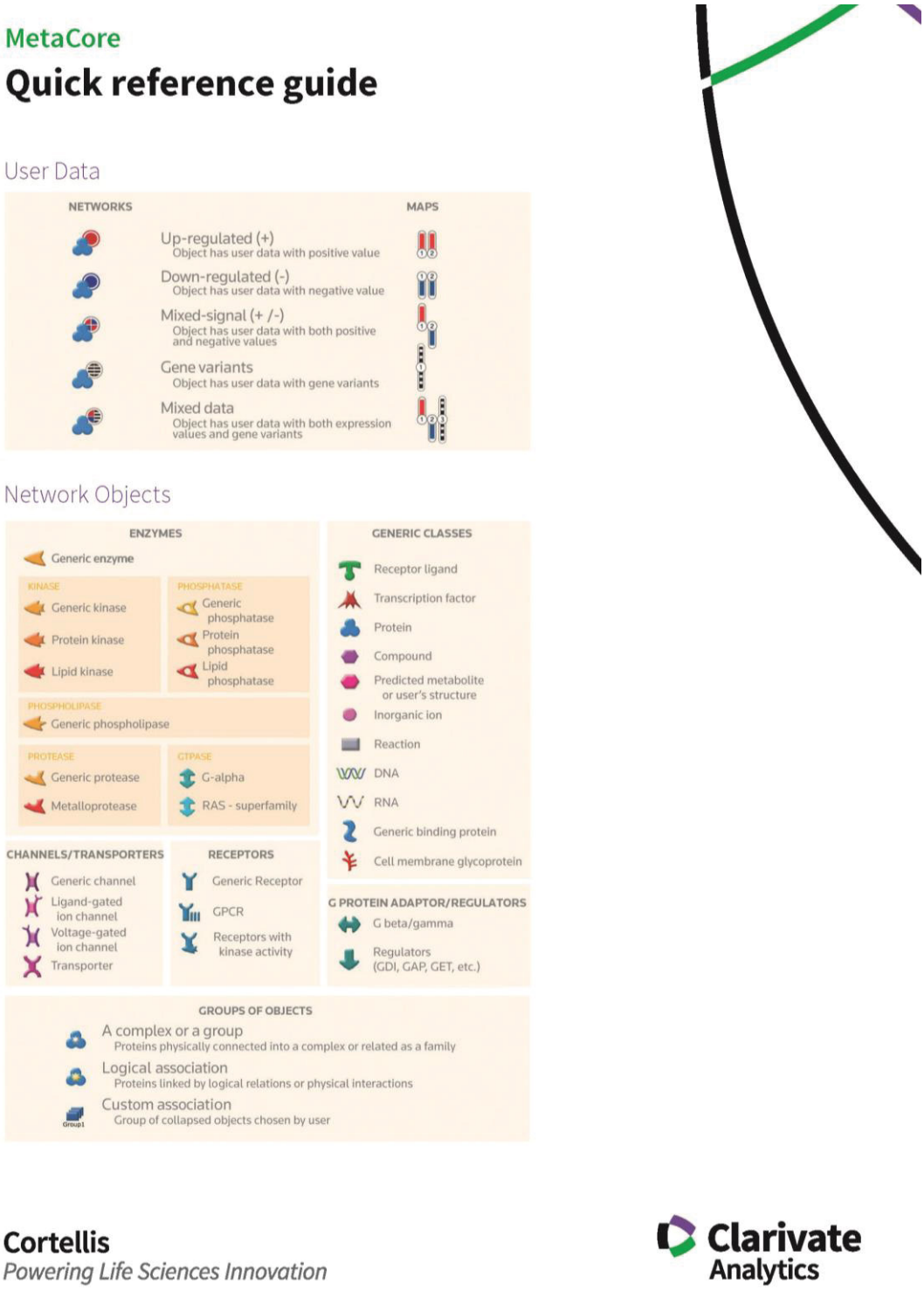
Additional information

Supplementary information is available for this paper.

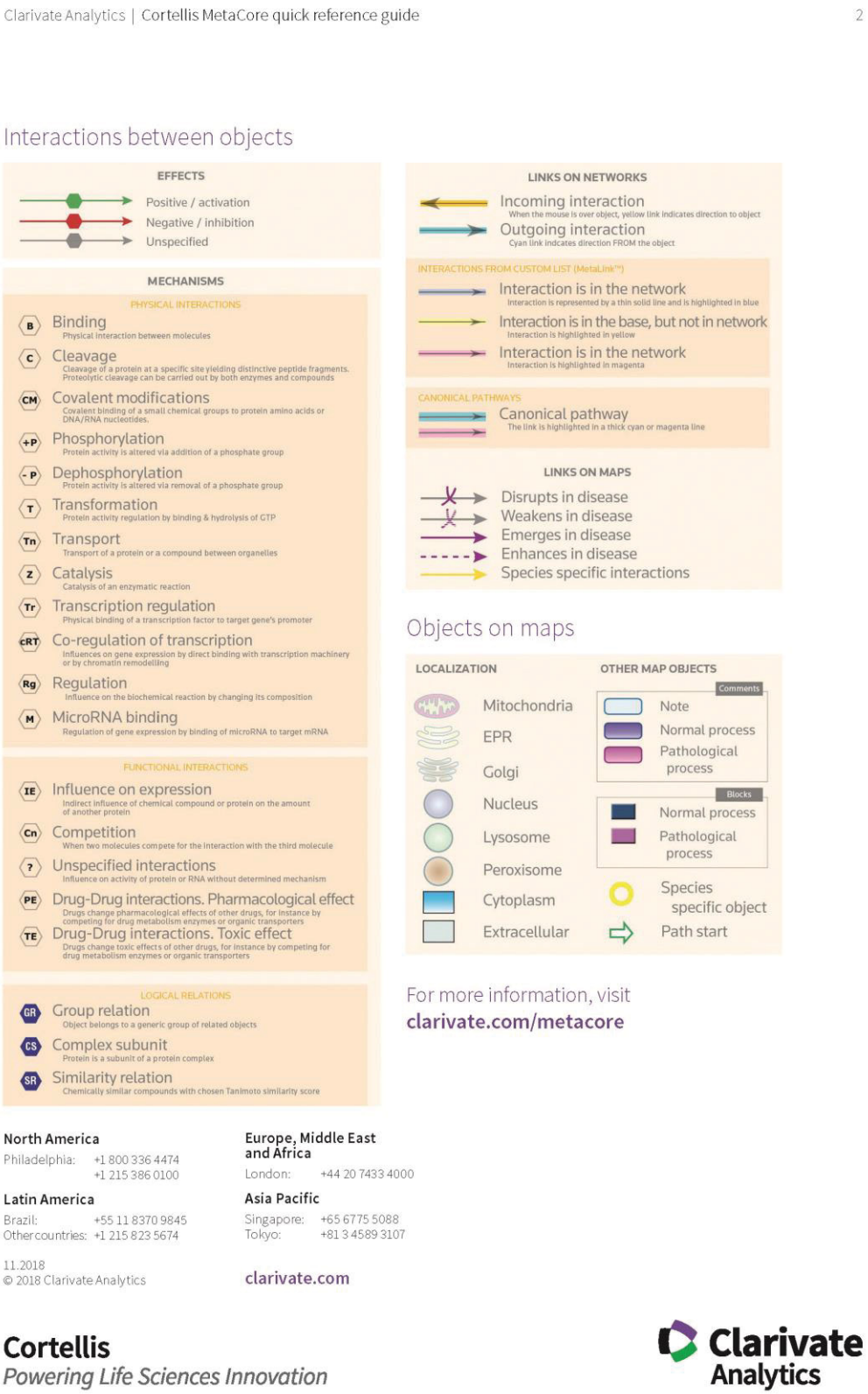
Supplementary information



Supplementary Fig. 1 | Altered gene expression in lungs of infected LTβR$^{-/-}$ mice. Preliminary RNAseq analysis of lung tissue of *T. gondii* infected (d7 *p.i.*) LTβR$^{-/-}$ mice (n=3) depicted as **a,** volcano plot and **b**, GSEA analysis. Dashed horizontal black line represents p=0.05. For RNAseq data mouse hyptothetical genes were excluded prior to conversion to gene counts. DEseq2 was used to test for differential expression and GO (Biological Process) molecular signatures were obtained using the msigdr package in R. Gene-level stats for each differential expression contrast were tested for geneset enrichment using the fgsea package, with an adjusted p-value of 0.1.



Supplementary Figure 2: MetaCore reference guide describing the different symbols and interactions depicted in network and pathway analysis.



Supplementary Figure 3: MetaCore reference guide describing the different symbols and interactions depicted in network and pathway analysis.

Supplementary Table 1: List of probes and primer sequences for amplification in qRT-PCR.

Gene	Probe [FAM]		Sequence [5' -> 3']
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
GTPBP1	75	fwd	GGTGCAGAGCAAAGATGATG
		rev	ATCTGGAATATCGGGCACAT
IFNγ	63	fwd	ATCTGGAGGAACTGGCAAAA
		rev	TTCAAGACTTCAAAGAGTCTGAGGTA
IFNβ	95	fwd	CAGGCAACCTTTAAGCATCAG
		rev	CCTTTGACCTTTCAAATGCAG
IL-12p40	27	fwd	GATTCAGACTCCAGGGGACA
		rev	TGGTTAGCTTCTGAGGACACATC
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	20	fwd	TGCCAACTTCCTCAGCTACA
		rev	GTGCACAGCAAAGTGATTGG
IDO1	2	fwd	GGGCTTCTTCCTCGTCTCTC
		rev	TGGATACAGTGGGGATTGCT
CD3	108	fwd	TCCCAACCCAGACTATGAGC
		rev	GCGATGTCTCTCCTATCTGTCA
CD4	99	fwd	AAAGAACTGGTTCGGCATGA
		rev	CGCTGACTCTCCCTCACTCT
CD8a	92	fwd	TGCTGTCCTTGATCATCACTCT
		rev	ACTAGCGGCCTGGGACAT
SOCS1	74	fwd	CAGCCGACAATGCGATCT
		rev	CGAAGACGAGGACGAGGA
SOCS3	83	fwd	ATTTCGCTTCGGGACTAGC
		rev	AACTTGCTGTGGGTGACCAT
β-actin	106	fwd	TGACAGGATGCAGAAGGAGA
		rev	CGCTCAGGAGGAGCAATG
TgB1	TgB1*	fwd	GCTAAAGGCGTCATTGCTGTT
		rev	GGCGGAACCAACGGAAAT

Primers (Metabion); numbered probes (Universal ProbeLibrary, Roche,); TM probes (TipMolBIOL); TgB1 probe (Metabion):
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: List of primary antibodies used in immunoblot analyses.

Primary antibodies	Host species	Supplier
anti-STAT1	rabbit	Cell Signaling Technology
anti-pSTAT (Tyr701)	rabbit	Cell Signaling Technology
anti-IRF-1 (D5E4) XP®	rabbit	Cell Signaling Technology
anti-mGBP2	rabbit	Eurogentec
anti-mGBP7	rabbit	Eurogentec
anti-β-actin (8H10D10)	mouse	Cell Signaling Technology
anti-GAPDH (14C10)	rabbit	Cell Signaling Technology

3.3 Indoleamine 2,3-Dioxygenase Activity During Acute Toxoplasmosis and the Suppressed T cell Proliferation in Mice

Authors

Christoph-Martin Ufermann, Andreas Domröse, Timo Babel, **Anne Tersteegen**, Sevgi Can Cengiz, Silvia Kathrin Eller, Katrin Spekker-Bosker, Ursula R. Sorg, Irmgard Förster, Walter Däubener

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Indoleamine 2,3-Dioxygenase Activity During Acute Toxoplasmosis and the Suppressed T Cell Proliferation in Mice

Christoph-Martin Ufermann[1†], Andreas Domröse[1†], Timo Babel[1], Anne Tersteegen[1], Sevgi Can Cengiz[2], Silvia Kathrin Eller[1], Katrin Spekker-Bosker[1], Ursula Regina Sorg[1], Irmgard Förster[2] and Walter Däubener[1]*

[1] Institute of Medical Microbiology and Hospital Hygiene, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany,
[2] Immunology and Environment, Life and Medical Sciences (LIMES) Institute, University of Bonn, Bonn, Germany

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Nicolas Blanchard,
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University of Wyoming, United States
Neide Maria Silva,
Federal University of Uberlandia, Brazil

***Correspondence:**
Walter Däubener
daeubene@uni-duesseldorf.de

†These authors have contributed equally to this work

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Toxoplasma gondii (*T. gondii*) is an obligate intracellular parasite and belongs to the phylum Apicomplexa. *T. gondii* is of medical and veterinary importance, because *T. gondii* causes the parasitic disease toxoplasmosis. In human cells, the interferon-gamma inducible indoleamine 2,3-dioxygenase 1 (IDO1) is an antimicrobial effector mechanism that degrades tryptophan to kynurenine and thus limits pathogen proliferation *in vitro*. Furthermore, IDO is described to have immunosuppressive properties, e.g., regulatory T cell differentiation and T cell suppression in humans and mice. However, there is only little known about the role of IDO1 in mice during acute toxoplasmosis. To shed further light on the role of mIDO1 *in vivo*, we have used a specifically adjusted experimental model. Therein, we infected mIDO1-deficient ($IDO^{-/-}$) C57BL/6 mice and appropriate wild-type (WT) control mice with a high dose of *T. gondii* ME49 tachyozoites (type II strain) *via* the intraperitoneal route and compared the phenotype of $IDO^{-/-}$ and WT mice during acute toxoplasmosis. During murine *T. gondii* infection, we found mIDO1 mRNA and mIDO1 protein, as well as mIDO1-mediated tryptophan degradation in lungs of WT mice. $IDO^{-/-}$ mice show no tryptophan degradation in the lung during infection. Even though *T. gondii* is tryptophan auxotroph and rapidly replicates during acute infection, the parasite load was similar in $IDO^{-/-}$ mice compared to WT mice 7 days post-infection. IDO1 is described to have immunosuppressive properties, and since T cell suppression is observed during acute toxoplasmosis, we analyzed the possible involvement of mIDO1. Here, we did not find differences in the intensity of *ex vivo* mitogen stimulated T cell proliferation between WT and $IDO^{-/-}$ mice. Concomitant nitric oxide synthase inhibition and interleukin-2 supplementation increased the T cell proliferation from both genotypes drastically, but not completely. In sum, we analyzed the involvement of mIDO1 during acute murine toxoplasmosis in our specifically adjusted experimental model and found

a definite mIDO1 induction. Nevertheless, mIDO1 seems to be functional redundant as an antiparasitic defense mechanism during acute toxoplasmosis in mice. Furthermore, we suggest that the systemic T cell suppression observed during acute toxoplasmosis is influenced by nitric oxide activity and IL-2 deprivation.

Keywords: *Toxoplasma gondii*, IDO, T cell suppression, mouse, kynurenine

INTRODUCTION

The apicomplexan parasite *Toxoplasma gondii* (*T. gondii*) is, due to the fact that it can infect nearly all warm-blooded animals, considered to be the most successful parasite worldwide. A primary *T. gondii* infection in humans is usually asymptomatic, but can cause congenital toxoplasmosis and can thus lead to fatal consequences for the fetus or newborn. In immunocompetent individuals, *T. gondii* establishes a chronic infection and will thus persist lifelong in the host. Reactivation of a chronic *T. gondii* infection in humans—e.g., during immunosuppression—results in cerebral toxoplasmosis in most cases (Schlüter et al., 2014). A recent study by Wilking et al. (2016) showed that *T. gondii* infection, while depending on demographic factors, is highly prevalent in Germany; about 55% of the representative cohort was seropositive for *T. gondii*.

Defense mechanisms directed against *T. gondii* are intensively studied. In addition, extensive data were obtained analyzing murine toxoplasmosis since the mouse model is the preferred animal model to study toxoplasmosis *in vivo* (Gazzinelli et al., 2014; Yarovinsky, 2014; Sasai et al., 2018).

Many different effector mechanisms are described to be involved in the defense against *T. gondii*, including iron depletion (Dimier and Bout, 1998) and enhanced autophagy (Krishnamurthy et al., 2017). However, the most frequently studied mechanisms directed against intracellular parasites in mice are the enhanced production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) (Adams et al., 1990; Khan et al., 1997) and the activity of GTPases, enzymes that can hydrolyze guanosine triphosphate (Hunn et al., 2011; Degrandi et al., 2013; Sasai et al., 2018). In humans, tryptophan depletion by indoleamine 2,3-dioxygenase (IDO) is the most frequently described defense mechanism (Pfefferkorn, 1984; MacKenzie et al., 2007).

iNOS was found to be effective against *T. gondii* in cell cultures, e.g., murine macrophages (Adams et al., 1990) or murine mesenchymal stem cells (Meisel et al., 2011), and more importantly, in *in vivo* studies using iNOS-deficient mice (Khan et al., 1997). However, in contrast to these findings, NO production favored the growth of *T. gondii* in cytokine-activated human uroepithelial cells (Däubener et al., 1999), human hepatocytes (Bando et al., 2018), and human retinal pigment epithelial cells (Spekker-Bosker et al., 2019).

Another important antiparasitic effector mechanism directed against *T. gondii* is the induction of GTPases such as immunity-related GTPases (IRGs) (Hunn et al., 2011) or murine guanylate binding proteins (mGBPs) (Degrandi et al., 2013; Sasai et al., 2018). In humans, there is only one IRG present, and this human IRG is not interferon inducible (Bekpen et al., 2005). Furthermore, human GBP-mediated antiparasitic mechanisms differ from those of murine GBPs (Hunn et al., 2011; Johnston et al., 2016).

The role of the interferon-gamma (IFN-γ) inducible IDO in the defense against *T. gondii* was first recognized *in vitro* using human fibroblasts (Pfefferkorn, 1984) and has been confirmed in other human cell lines (e.g., epithelial and endothelial cells) (MacKenzie et al., 2007). In contrast, in murine cells, mIDO does not mediate defense against intracellular *T. gondii* tachyzoites after IFN-γ stimulation as shown in macrophages and mesenchymal stroma cells (Schwartzman et al., 1990; Meisel et al., 2011). Interestingly, another isoform of IDO has been reported, named indoleamine 2,3-dioxygenase 2 (IDO2). IDO2 has a lower tryptophan affinity than IDO1 *in vitro*, and its induction, expression, as well as distribution are described to be different from IDO1 (Yeung et al., 2015).

Despite the abovementioned differences in the IDO-mediated antiparasitic effects between human and murine cells, IDO-mediated immunoregulatory effects have been described in both murine and human cells. For example, the group of Munn and coworkers found an important function for IDO in the development of immune tolerance in allogeneic pregnancy in mice and described a profound immunosuppression mediated by IDO-positive human macrophages (Munn et al., 1998, 1999). Furthermore, they described the tryptophan depletion as a possible reason for the inhibited T cell proliferation (Munn et al., 1998, 1999); thus, we suggest that this local reduction of tryptophan could also cause a local antimicrobial environment. The immunosuppressive activity of IDO has been confirmed by several groups and is of interest in transplant medicine as well as in tumor immunology and autoimmunity as reviewed previously (Löb et al., 2009). In sum, it was found that IDO-positive dendritic cells (DCs) are able to induce tolerance during T cell activation, while within the tissue, IDO-positive non-professional antigen-presenting cells such as fibroblasts and

Abbreviations: ConA, concanavalin A; CpG B, class B phosphate linked cytosine and guanine oligonucleotide; DC, dendritic cell; dpi, days post-infection; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; GBP, guanylate binding protein; GTPase, enzyme that hydrolyzes guanosine triphosphate; HFF, human foreskin fibroblasts; HPLC, high-performance liquid chromatography; i. p., intraperitoneal; IDO, indoleamine 2,3-dioxygenase; IL-2, interleukin-2; IMDM, Isocove's Modified Dulbecco's Medium; inf, infected; IFN-γ, interferon gamma; iNOS, inducible nitric oxide synthase; IRG, immunity-related GTPase; MLN, mesenteric lymph node; n.d., not detectable; n.s., not significant; N^GMMA, N^G-monomethyl-L-arginine; NO, nitric oxide; PBS, phosphate-buffered saline; qRT PCR, quantitative real-time polymerase chain reaction; SD, standard deviation; SEM, standard error of the mean; TDO, tryptophan 2,3-dioxygenase; WT, wild type; 1-L-MT, 1-methyl-L-tryptophan.

endothelial cells could inhibit the effector function of T cells (Lee et al., 2017).

Since the aforementioned effector mechanisms directed against *T. gondii* are differentially regulated in different species as well as cell types, we decided to investigate the influence of IDO on acute toxoplasmosis in a specifically adjusted murine experimental *in vivo* model.

MATERIALS AND METHODS

Cell Line and Parasite Strain Cultivation

Human foreskin fibroblasts (HFF; ATCC® SCRC-1041™, Wesel, Germany) and the murine macrophage cell line (RAW 264.7; ATCC® TIB-71™, Wesel, Germany) were cultured in Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Carlsbad, USA), supplemented with 5% (vol/vol) heat-inactivated fetal bovine serum (FBS; BioWhittaker®, Lot N°: 9SB003, Lonza, Basel, Switzerland). Cells, as well as isolated cells for *ex vivo* cultivation, were kept in a humidified Heraeus BB 6220 CO_2 incubator (Thermo Fisher Scientific, Waltham, USA) (37°C, 5% CO_2). HFF cells were passaged after confluency was reached using 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA), (Life Technologies, Carlsbad, USA). Confluent HFF monolayers were used as host cells.

T. gondii strain ME49 tachyzoites (ATCC® 50611, Wesel, Germany) were maintained *in vitro* by serial passages in HFF. For infection experiments, parasites were propagated in HFF (for 42–48 h). Parasites were harvested by scraping off parasitized HFFs in phosphate-buffered saline (PBS) (Life Technologies, Carlsbad, USA). Intracellular parasites were syringe-released and dissociated from host cells debris by differential centrifugation [85×*g*, room temperature (RT), 5 min; 780×*g*, RT, 5 min]. Parasites were resuspended in PBS, counted, and adjusted to 5 × 10^5 tachyzoites/ml.

Animals and Infection Experiments

mIDO1-deficient ($IDO^{-/-}$) mice (B6.129-*Ido1*^{tm1Alm}/J) were originally obtained from the Jackson Laboratory (Bar Harbor, Maine, USA) and had a C57BL/6 genetic background. $IDO^{-/-}$ mice were bred and kept under specific pathogen-free (SPF) conditions in the Central Unit for Animal Research and Animal Welfare Affairs of the Heinrich-Heine-University Düsseldorf. C57BL/6 (C57BL/6JRj) mice purchased from Janvier Labs (Le Genest-Saint-Isle, France) were used as wild-type (WT) controls. All experiments were performed with age- and sex-matched cohorts. Mice were infected intraperitoneally (i.p.) with 10^5 *T. gondii* ME49 tachyzoites in 200 µl of PBS. Naïve control mice and infected mice were kept under SPF conditions and were checked daily. For sample collection, mice were euthanized by cervical dislocation 7 days post-infection (dpi). This study was performed in strict compliance with the German Animal Welfare Act. The experiments were authorized by the North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection (Permit# 84-02.04.2013.A271, 84-02.04.2013.A495, and 84-02.04.2016.A508). All efforts were made to minimize animal suffering during the experiments.

Sample Collection

Blood samples were taken by cardiac puncture, and the sera were generated from clotted blood samples (4°C overnight) in two centrifugation steps (20,000 × *g*, 4°C, 10 min). Organs [lung, brain, liver, spleen, and mesenteric lymph nodes (MLNs)] were collected and washed in PBS. Whole lung, brain, and liver were homogenized in PBS using the Percellys® lysing kit CK28 and the Percellys® Minilys® tissue homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France). All samples were stored at −80°C for further processing.

Western Blot Analyses

The protein contents of supernatants from centrifuged tissue homogenates or cell lysates generated by freeze–thaw were determined *via* the Bradford assay (Bio-Rad Laboratories, Hercules, USA). Electrophoretic separation of proteins (30 µg protein per lane) was done with 10% NuPAGE Novex Bis-Tris Mini gels in the appropriate electrophoresis system (Thermo Fisher Scientific, Waltham, USA). Proteins were semi-dry blotted on nitrocellulose membranes (CarboGlas, Schleicher & Schüll, Dassel, Germany). Membranes were blocked in 5% (w/v) skim milk powder in PBS for 1 h at RT. For specific protein detection, the primary antibodies for murine β-actin (1:10,000) (AC-15, Sigma-Aldrich, Munich, Germany), murine iNOS (1:1,000) (1131-1144, CalBiochem®, Munich, Germany), or murine IDO (1:500) (AB9900, Chemicon, Merck Millipore, Billerica, MA, USA) were diluted in 0.5% (w/v) skim milk powder in PBS. Membranes were incubated for 1.5 h at RT and were washed three times with PBS (5 min each). The peroxidase-conjugated, secondary antibodies goat anti-mouse IgG (for mβ-actin) or goat anti-rabbit IgG (for mIDO and miNOS) (1:10,000–70,000, Jackson ImmunoResearch Laboratories, Dianova, Hamburg, Germany) were diluted in 0.5% (w/v) skim milk powder in PBS. Membranes were incubated for 2 h at RT and were washed three times with PBS (5 min each). Labeled proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Freiburg, Germany).

qRT-PCR Analysis of Transcript Levels

Total RNA was extracted according to the TRI Reagent protocol (Merck, Darmstadt, Germany). Briefly, total RNA was extracted from 50 µl of lung tissue homogenate with 500 µl of TRI Reagent and 100 µl of chloroform followed by precipitation with isopropyl alcohol. Extracted RNA was dissolved in 40 µl of UltraPure™ distilled water (Thermo Fisher Scientific, Waltham, USA) and RNA concentration was determined *via* NanoDrop (Thermo Fisher Scientific, Waltham, USA). Reverse transcription of 1.5 µg of total RNA to cDNA was performed with M-MLV reverse transcriptase and oligo(dT) 12–18 primers according to the manufacturer's instruction (Thermo Fisher Scientific, Waltham, USA). PCR primers to amplify the genes of interest were designed using the Universal ProbeLibrary Assay Design Center (Roche, Basel, Switzerland) and are listed in **Supplementary Table S1**. Real-time PCR was performed with the Takyon NoRox Probe MasterMix dTTP (Eurogentec, Lüttich, Belgium) on a BioRad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, USA). Quality of qPCR

analysis was verified by technical replicates for each sample in each run. Each well of a multiplate 96-well PCR plate contained 5 µl of cDNA template, 12.5 µl of Takyon NoRox Probe Master Mix dTTP, 0.3 µl of primer (10 µM each), 0.5 µl of probe (10 µM), and 6.4 µl of H_2O for a total reaction volume of 25 µl. The PCR conditions were 7 min at 95°C and 40 cycles of 95°C for 20 s and 60°C for 1 min.

Tryptophan and Kynurenine Quantification

We used high-performance liquid chromatography (HPLC) analysis to quantify total free tryptophan and kynurenine in mice serum and lung tissue. To precipitate existing proteins within the samples, they were mixed with trichloroacetic acid (2.5% final concentration; Sigma-Aldrich, Munich, Germany). To monitor measurement quality, all samples were mixed with 3-nitro-L-tyrosine (Sigma-Aldrich, Munich, Germany) with final concentrations of 2.5 or 10 µg/ml for lung tissues or sera, respectively, as internal standard. All samples were filtered (pore size 0.22 µm) prior to injection.

Analysis was performed with a System Gold® HPLC system (Beckman Coulter, Krefeld, Germany) under usage of a module 166 UV/VIS detector. For separation, a reverse-phase C18 column cartridge (Purospher® STAR RP-18 endcapped, Sorbent Lot No. FC095368, 3-µm particle size, 55-mm length, 2-mm diameter; Merck, Darmstadt, Germany) with an adequate guard column (Purospher® STAR RP-18 endcapped, Sorbent Lot No. HX435803, 5 µm particle size, 4 mm length, 4 mm diameter; Merck, Darmstadt, Germany) was used in a manuCART® 55-mm cartridge holder (Merck, Darmstadt, Germany). The mobile phase consisted of 50 mM sodium acetate (Merck, Darmstadt, Germany) adjusted to pH 4.2 with acetic acid (Merck, Darmstadt, Germany) with 5 or 2% acetonitrile (Merck, Darmstadt, Germany) for tryptophan and kynurenine analysis, respectively, using a flow of 0.5 ml/min. All eluents were purchased at least as gradient grade and underwent a vacuum degassing as well as a filtration with a 2 µm filter. The absorbance was measured at 280 nm for tryptophan and 360 nm for kynurenine; calculation occurred on the basis of previously measured calibration curves with purchased highly pure L-tryptophan and L-kynurenine (Sigma-Aldrich, Munich, Germany).

qPCR Analysis of the Parasite Load

DNA was extracted from lung tissue homogenate by proteinase K digestion. In brief, 500 µl of digestion buffer (1% proteinase K (200 µg/mL; Qiagen, Venlo, Netherlands) in lysis buffer [100 mM Tris/HCl (pH 8.5), 5 mM EDTA (pH 8), 0.2% SDS, and 200 mM NaCl] was added to 20 µl of lung tissue homogenate and was incubated at 56°C and 1,100 rpm on a thermo-shaker for 90 min. DNA was precipitated with 500 µl of isopropyl alcohol and washed with 500 µl of 70% ethanol. Extracted DNA was dissolved in 50 µl of UltraPure™ distilled water and the DNA concentration was adjusted to 100 ng/µl. Quantitative real-time PCR (qPCR) was performed with the abovementioned detection system. For parasite quantification, a standard curve with adjusted *T. gondii* genomic DNA concentrations was established. The oligonucleotides and template-specific probe that were used are listed in **Supplementary Table S1**. These oligonucleotides bind to a sequence segment of the 35-fold repetitive B1 gene of *T. gondii* that is commonly used in diagnostics (Burg et al., 1989; Pelloux et al., 1998). Quality of qPCR analysis was verified by technical replicates for each sample in each run. Each well of a multiplate 96-well PCR plate contained 5 µl of DNA template, 12.5 µl of Takyon NoRox Probe Master Mix dTTP, 2.5 µl of primer (3 µM each), and 2.5 µl of probe (2 µM) for a total reaction volume of 25 µl. The PCR conditions were 10 min at 95°C and 45 cycles of 95°C for 15 s and 60°C for 1 min.

Isolation and Cultivation of Murine Cells From Spleen and Mesenteric Lymph Nodes

For *ex vivo* lymphocyte proliferation experiments, cells from spleen and MLN tissues were digested using 1 mg/ml collagenase (C2139, Sigma-Aldrich, Munich, Germany) and 180 U/ml DNase (Roche, Basel, Switzerland) in PBS for 30 min at 37°C. Digested tissues were passed through 70-µm nylon sieves (Falcon® Corning Inc.; Corning, New York, USA) followed by erythrocyte lyses (MORPHISTO GmbH; Frankfurt am Main, Germany). Cells were resuspended in medium [IMDM with 5% FBS and 100 U/ml penicillin/100 µg/ml streptomycin (Biochrom GmbH, Berlin, Germany)] and counted using trypan blue (0.4%; Sigma-Aldrich, Munich, Germany). Cells were seeded in low-evaporation lid 96-well flat-bottom plates (Corning Inc., Corning, New York, USA) at 3×10^5 cells per well.

Lymphocyte Proliferation Assay

Lymphocyte proliferation was stimulated with the mitogens concanavalin A (ConA; 1 µg/ml; Sigma-Aldrich, Munich, Germany) and the class B phosphate-linked cytosine and guanine oligonucleotide ODN1826 (CpG B; 0.1 µM; Invivogen; San Diego, CA, USA) as indicated. Additional supplementation with recombinant human interleukin-2 (IL-2; 5 ng/mL; R&D Systems, Minnesota, USA) and the NOS inhibitor N^G-monomethyl-L-arginine (N^GMMA; 100 µg/ml; Merck, Darmstadt, Germany) was performed in the concentrations indicated.

Lymphocyte proliferation was determined by the ^{3}H-thymidine incorporation method. In brief, ^{3}H-thymidine (74 kBq per well; GE Healthcare Buchler GmbH & Co. KG, Braunschweig, Germany) was added 48 h post-stimulation. Lymphocyte proliferation was stopped after additional 24 h of cultivation by freezing. Lymphocyte proliferation was determined by measuring incorporated ^{3}H-thymidine using liquid scintillation spectrometry (1205 Betaplate, PerkinElmer, Jugesheim, Germany).

Indirect Nitric Oxide Estimation

NO production was measured *via* the Griess assay (Ding et al., 1988). Here, nitrite—a stable breakdown product of NO—is measured. In brief, 100 µl of cell culture supernatant was used after 72 h of *in vitro* cultivation. The Griess assay was performed as described before (Meisel et al., 2011). The nitrite content was calculated by extrapolation from a sodium nitrite standard curve assayed parallel to each measurement.

Statistical Analysis

Results are indicated as means ± SD or ± SEM as indicated in figure legends. Statistical significances of differences in mean values were analyzed by using the unpaired two-tailed Student's *t*-test (GraphPad Prism). Significant differences were indicated with asterisks ($^{*}p \leq 0.05$; $^{**}p \leq 0.001$; $^{***}p \leq 0.0001$).

RESULTS

Expression of mIDO1 mRNA and Protein in Lungs of Infected Mice

To clear up potentially different murine indoleamine 2,3-dioxygenase 1 (mIDO1) distribution among various murine tissues within WT and IDO$^{-/-}$ mice, Western blot analyses were performed. Here, no mIDO protein was detectable in liver, brain, or lung tissue of naïve WT mice, while infection induced strong mIDO1 expression in lung tissue as well as a slight expression in liver tissue. As suggested in IDO$^{-/-}$ mice, in all tested conditions and tissues, no mIDO protein was detectable (**Figure 1A**). Further quantitative real-time PCR experiments with the lung tissues were conducted to detect mIDO1 mRNA. Shown data represent the relative gene expression of infected to naïve mice in WT and IDO$^{-/-}$, respectively. Expression of mGBP2 was equally strong in WT and IDO$^{-/-}$ mice 7 dpi, as expected. Upon *T. gondii* infection, mIDO1 mRNA expression is strongly upregulated in WT mice. As expected, we did not detect any mIDO1 expression in infected IDO$^{-/-}$ mice. Murine mIDO2 was measured as well to exclude mIDO2 as a responsible candidate for results shown further on. mIDO2 is only marginally increased during *T. gondii* infection in a few infected mice and there is no significant difference between WT and IDO$^{-/-}$ mice (**Figure 1B**). Additionally, we measured miNOS expression in lungs of WT and IDO$^{-/-}$ mice at different time points post-infection *via* quantitative real-time PCR and Western blot analysis (**Supplementary Figure S1**). Relative miNOS expression in infected WT mice was increased in a time-dependent manner (**Supplementary Figure S1A**). However, differences between WT and IDO$^{-/-}$ on day 7 post-infection were not significant (**Supplementary Figure S1B**). miNOS protein was absent in lungs of naïve WT and IDO$^{-/-}$ mice. Infected IDO$^{-/-}$ mice were positive for miNOS protein at 7 and 9 dpi and infected WT mice only at 9 dpi (**Supplementary Figure S1C**).

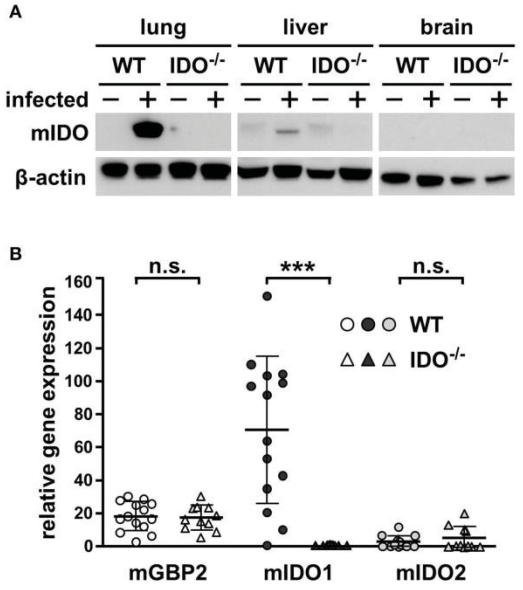

FIGURE 1 | Expression of mIDO1 in murine lung tissue during *Toxoplasma gondii* infection. Gene and protein expression in tissues from naïve or *T. gondii* ME49-infected wild-type (WT) and indoleamine 2,3-dioxygenase 1-deficient (IDO$^{-/-}$) mice. Western blot analysis shows murine IDO (mIDO) and β-actin protein in lung, liver, and brain tissue of naïve (–) and infected (+) WT and IDO$^{-/-}$ mice **(A)**. Expression of murine guanylate binding protein 2 (mGBP2), mIDO1, and mIDO2 in lung tissue homogenates of infected mice relative to their expression in naïve control samples **(B)**. Data were normalized to the housekeeping gene β-actin and were represented as $2^{-\Delta\Delta CT}$ (naïve vs. infected) in scattered dot plots and means ± standard deviation. The Student's *t*-test (unpaired, two-tailed) was used to determine statistical differences marked with asterisks (n.s., not significant; $^{***}p \leq 0.0001$).

Comparison of mIDO1-Based Tryptophan Degradation and Parasite Loads of Naïve and Infected WT and IDO$^{-/-}$ Animals

We explored the antiparasitic properties influenced by mIDO1 during *T. gondii* infection by comparing the tryptophan degradation as well as the parasite load.

Therefore, we determined tryptophan and kynurenine concentrations in sera *via* HPLC analyses to analyze the systemic distribution of these metabolites. Furthermore, we analyzed lung tissue *via* HPLC, since we previously identified lung tissue as one center of mIDO1 protein and mRNA expression.

With 15.3 and 17.7 μg/ml, naïve WT and IDO$^{-/-}$ mice exhibit no significant differences in mean tryptophan concentrations in serum. On day 7 after *T. gondii* infection, serum tryptophan concentrations drop significantly to 7.5 and 10.2 μg/ml in WT and IDO$^{-/-}$ animals, respectively. Concomitantly with this serum tryptophan drop, the serum kynurenine concentration in the WT rises significantly from 0.23 to 0.94 μg/ml. Even though the serum tryptophan concentration drops in the IDO$^{-/-}$ animals similar to the WT, the serum kynurenine concentration is unaltered (<0.1 μg/ml) in the IDO$^{-/-}$ animals (**Figure 2A**). The lung tryptophan concentrations of naïve WT and IDO$^{-/-}$ mice behaved like the serum tryptophan concentrations without significant differences, but with 7.7 and 7.3 μg/ml, they are overall lower. Infected WT animals show a significant drop in lung tryptophan concentration (from 7.7 to 2.4 μg/ml) paired with a significant increase in the lung kynurenine concentration (from 0.4 to 6.7 μg/ml). In contrast, infected IDO$^{-/-}$ animals exhibit no significant difference in lung tryptophan concentrations compared with the naïve group. Kynurenine concentrations in lungs of naïve and infected IDO$^{-/-}$ animals are likewise low (<0.1 μg/ml) as in sera (**Figure 2B**).

To draw a conclusion regarding the previously mentioned potential antiparasitic properties of mIDO1, we determined

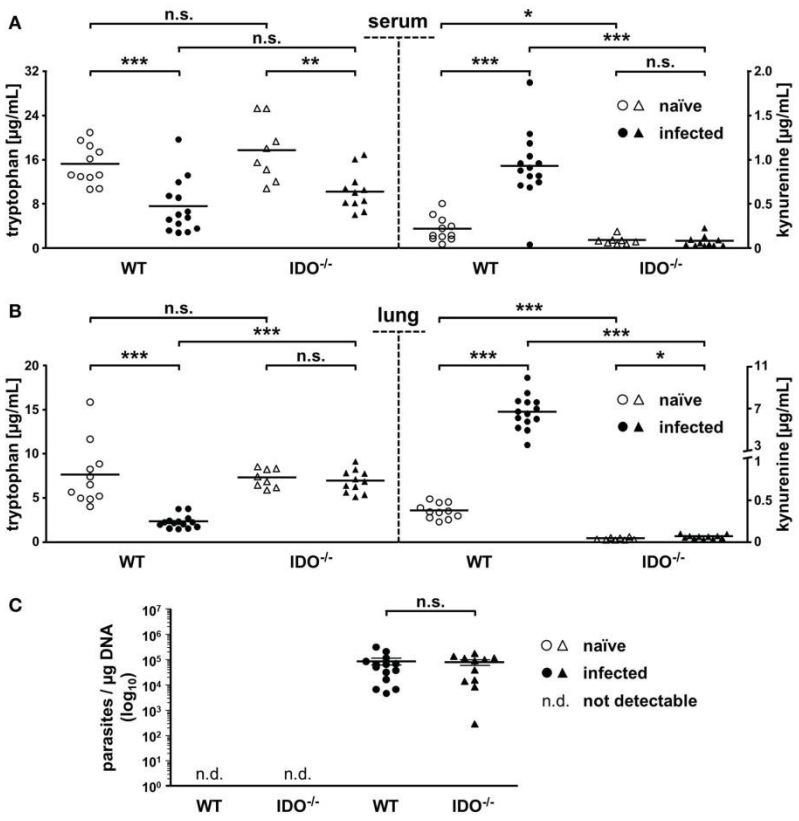

FIGURE 2 | mIDO1 activity in murine lung tissue during *Toxoplasma gondii* infection. Serum and lung samples were collected from naïve or *T. gondii* ME49-infected wild-type (WT) and indoleamine 2,3-dioxygenase 1-deficient ($IDO^{-/-}$) mice. Free tryptophan and kynurenine was measured *via* high-performance liquid chromatography in serum **(A)** and lung tissue homogenate samples **(B)**. Comparison of the *T. gondii* load (determined by B1-gene detection) in lungs of naïve or infected WT and $IDO^{-/-}$ mice **(C)**. Data are represented as scattered dot plots and means (±standard error of the mean for RT-PCR data). For the RT-PCR data, each dot represents the mean of duplicate measurements of a single mouse lung tissue sample. The Student's *t*-test (unpaired, two-tailed) was used to determine statistical differences marked with asterisks (n.s., not significant; $^{*}p \leq 0.05$, $^{**}p \leq 0.001$, and $^{***}p \leq 0.0001$).

the parasite load in lung tissues *via* real-time PCR from the same samples we analyzed beforehand. Here, we used specific oligonucleotides to detect the 35-fold repetitive B1 gene of *T. gondii*. As expected, there was no detection of *T. gondii* in naïve WT and $IDO^{-/-}$ mice. We measured a significant amount of parasites in the lungs of WT and $IDO^{-/-}$ mice 7 dpi; however, the parasite load in WT and $IDO^{-/-}$ mice was not significantly different (**Figure 2C**).

Suppressed T Cell Proliferation Responses During Acute *T. gondii* Infection

Splenocytes were isolated from naïve and infected WT mice to analyze the proliferative responses of lymphocytes during acute toxoplasmosis. We performed initial T cell proliferation experiments to analyze the suitability of our specifically adjusted experimental model (**Supplementary Figure S2**). Therefore, we infected WT mice i.p. with *in vitro* cultivated tachyzoites or bradyzoites isolated from lysed brain cysts propagated *in vivo*. Here, we could not detect any differences in mitogen-stimulated T cell proliferation responses (**Supplementary Figure S2A**). Furthermore, we tested the time-dependent mitogen-stimulated T cell proliferation responses. The results shown in **Supplementary Figure S2B** clearly illustrate that the proliferation responses are not impaired at 3 dpi, are reversibly impaired at 7 dpi, and are irreversibly impaired at 10 dpi (**Supplementary Figure S2**). The proliferation of T cells and B cells was induced by stimulation with the mitogens concanavalin A (ConA) and the class B CpG oligonucleotide ODN1826, respectively (**Figure 3A**). Untreated splenocytes from infected WT mice show a weak basal proliferation compared to splenocytes from naïve mice. Mitogen stimulation of naïve splenocytes induced a potent lymphocyte proliferation response, whereas splenocytes from infected mice have a low proliferation response. In more detail, CpG B stimulation showed that the B cell proliferation response was slightly reduced by approximately 28% during acute *T. gondii* infection. However, T

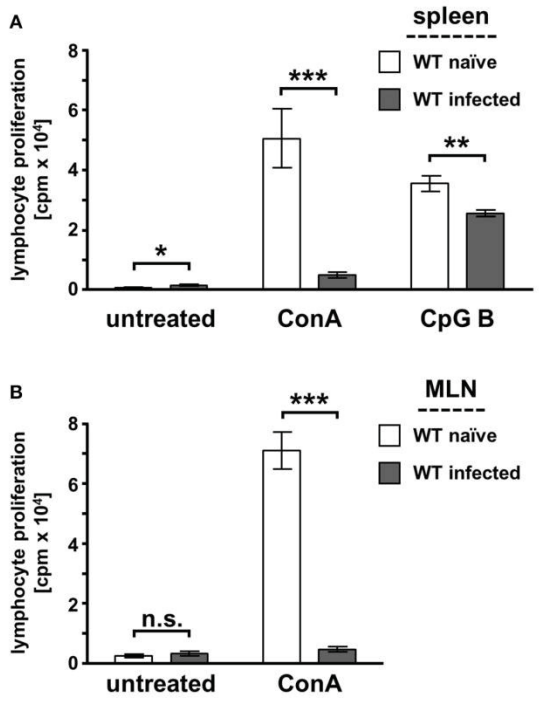

FIGURE 3 | Mitogen-stimulated lymphocyte proliferation is impaired during acute toxoplasmosis in WT spleen and MLN-derived cells. Spleen **(A)** and mesenteric lymph nodes (MLN) **(B)** were isolated from naïve or *T. gondii* ME49-infected wild-type (WT) mice. Cell cultures were supplemented with the mitogen concanavalin A (ConA; 1 μg/ml), the class B phosphate linked cytosine, and guanine oligonucleotide ODN1826 (CpG B; 0.1 μM) to stimulate T cell and B cell proliferation *ex vivo* or were left untreated. Lymphocyte proliferation was determined with the ^{3}H-thymidine method. Data were represented as means of triplicate measurements ($n = 7$–9) ± standard error of the mean. The Student's *t*-test (unpaired, two-tailed) was used to determine statistical differences marked with asterisks (n.s., not significant; $^{*}p \leq 0.05$, $^{**}p \leq 0.001$ and $^{***}p \leq 0.0001$).

cell stimulation with ConA showed a very prominent impaired T cell proliferation response (>90% reduction). ConA stimulation of MLN cells from naïve and infected WT mice resulted in the same phenotype (**Figure 3B**). In detail, MLN-derived T cells also showed a very weak proliferative response to *ex vivo* ConA stimulation during acute *T. gondii* infection (>90% reduction) as observed in the stimulation of splenic T cells. Thus, further experiments were conducted with splenocytes to perform more profound analyses of the T cell responses during acute toxoplasmosis.

IL-2 Availability and NOS Activity, but Not mIDO1 Influence T Cell Proliferation Responses During Acute Toxoplasmosis

The role of mIDO1 in the suppressed T cell proliferation responses during acute toxoplasmosis is not known. Thus, splenocytes were isolated from naïve and *T. gondii*-infected WT and IDO$^{-/-}$ mice 7 dpi.

Splenic T cells from naïve WT and IDO$^{-/-}$ mice respond comparably strong to mitogen stimulation (**Figure 4A**). During acute *T. gondii* infection, the mitogen-induced proliferative responses were highly reduced in WT splenocytes (>90%) and IDO$^{-/-}$ splenocytes (>92%) (**Figure 4A**). This indicates that the suppressed T cell responses are affected independently of mIDO1.

IL-2 deprivation (Khan et al., 1996) as well as iNOS activity (Patton et al., 2002) have previously been described to be involved in the impaired T cell proliferation response during acute toxoplasmosis. Thus, we performed *ex vivo* mitogen stimulation experiments with supplementation of IL-2 and the NOS inhibitor N^{G}MMA to elucidate their interplay in the proliferation of splenocytes from WT and IDO$^{-/-}$ mice. Supplementation of IL-2 alone did not significantly improve the proliferation of T cells from either infected genotype (WT: from 9.7 to 14.1%; IDO$^{-/-}$: from 7.6 to 10.9%) (**Figure 4A**). NOS inhibition *via ex vivo* N^{G}MMA treatment resulted in a small but significant elevation of T cell proliferation, which was equally strong for cells from both infected genotypes (WT: from 9.7 to 29.7%; IDO$^{-/-}$: from 7.6 to 30.2%) (**Figure 4A**). Combining the supplementation of IL-2 and N^{G}MMA increased the proliferation of mitogen-treated T cells from both infected genotypes even further, without however reaching the level of the naïve proliferation response (WT: from 9.7 to 59.5%; IDO$^{-/-}$: from 7.6 to 61.8%) (**Figure 4A**), thus resulting in a highly significant elevation of the proliferative response compared to mitogen stimulation alone.

Stimulated as well as untreated splenocytes isolated from *T gondii*-infected WT and IDO$^{-/-}$ mice showed NOS activity as measured indirectly *via* nitrite accumulation in the supernatant (**Figure 4B**). Here, splenocytes from IDO$^{-/-}$ mice produce significantly more nitrite compared to the equally treated WT splenocytes (untreated: 4.6 μM for WT and 8.1 μM for IDO$^{-/-}$; ConA stimulated: 5.9 μM for WT and 9 μM for IDO$^{-/-}$) (**Figure 4B**). NOS inhibition *via ex vivo* N^{G}MMA treatment reduced the nitrite concentration in supernatants strongly to 1.4 and 2.2 μM for WT and IDO$^{-/-}$, respectively (**Figure 4B**).

DISCUSSION

Indoleamine 2,3-dioxygenase (IDO) is described as a potent antimicrobial factor in *in vitro* systems using human, porcine, and bovine cells. In this context, IDO activity has been shown to inhibit pathogens like bacteria (e.g., group A streptococci, *Staphylococcus aureus*), viruses (e.g., Herpes simplex virus 1, Cytomegalovirus), and parasites (e.g., *T. gondii, Neospora caninum*) (Däubener et al., 2009). However, the role of IDO as a potent antimicrobial factor *in vivo* remains controversial. Here, we used C57BL/6 mice deficient for mIDO1 (IDO$^{-/-}$) to investigate acute toxoplasmosis with specific regard to the general systemic proinflammatory reaction and the local parasite burden within the lung, a strong IDO-expressing organ. We have adjusted our experimental model by comparing the infection with tachyzoites and bradyzoites. Furthermore, we have

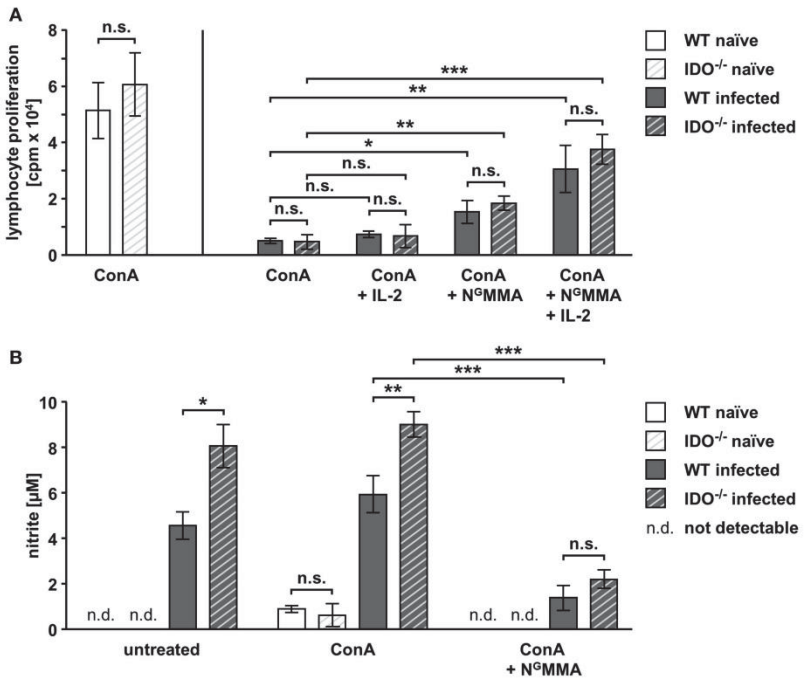

FIGURE 4 | T cell proliferation is altered by IL-2 and NOS activity in splenocytes derived from WT and IDO$^{-/-}$ mice. Splenocytes were isolated from naïve or *T. gondii* ME49-infected wild-type (WT) and indoleamine 2,3-dioxygenase 1-deficient (IDO$^{-/-}$) mice (WT naïve: $n = 7$; IDO$^{-/-}$ naïve: $n = 5$; WT infected: $n = 8$; IDO$^{-/-}$ infected: $n = 10$). Splenic T cell cultures were stimulated with the mitogen concanavalin A (ConA, 1 µg/ml) *ex vivo*. Supplementation of human interleukin-2 (IL-2, 5 ng/ml) and the nitric oxide synthase (NOS) inhibitor N^{G}-monomethyl-L-arginine (N^{G}MMA, 100 µg/ml) was done as indicated **(A,B)**. Lymphocyte proliferation was determined with the ^{3}H-thymidine method. **(A)**. Nitrite accumulation in the supernatant of *ex vivo* cultured splenocytes was detected using the Griess reaction **(B)**. Data were represented as means of triplicate measurements ± standard error of the mean. The Student's *t*-test (unpaired, two-tailed) was used to determine statistical differences marked with asterisks (n.s. = not significant; $^{*}p \leq 0.05$, $^{**}p \leq 0.001$, and $^{***}p \leq 0.0001$).

evaluated the optimal time point for our objectives. In this specifically adjusted experimental model, using a high dose of tachyzoites *via* the intraperitoneal route, C57BL/6 mice develop a more intense acute toxoplasmosis compared to BALB/c mice (own preliminary data not shown). The type II strain *T. gondii* ME49 was chosen for our infection experiments, since type II strains are the most frequently found *T. gondii* strains in human toxoplasmosis (Schlüter et al., 2014). To ensure standardized infection inoculums, we infected mice with a high dose of *T. gondii* ME49 tachyzoites *via* i.p. injection, thereby circumventing the use of brain-derived cysts that vary in size and parasite number (Dubey and Frenkel, 1998). However, it has to be taken into account that oral cyst uptake is a natural route of infection, whereas i.p. injection of tachyzoites into inbred mice is a strictly experimental setup. Additionally, infection *via* the natural route results in a slower but more natural course of disease compared to our specifically adjusted experimental model. Finally, it has to be considered that our model does not represent the natural course of toxoplasmosis, but is ideally adjusted for the herein analyzed objectives. Furthermore, it cannot be excluded that the high infection dose used in our model might mask a role of IDO during the natural course of a *T. gondii* infection.

Here, we show that infection of mice with *T. gondii* tachyzoites results in a strong mIDO1 induction in lungs. In detail, we found high amounts of mIDO1 mRNA and mIDO protein in the lungs of *T. gondii*-infected WT animals. Similar observations have been obtained during allergic diseases (Hayashi et al., 2004) or allogeneic stem cell transplantation (Lee et al., 2017) in mice. In both publications, mIDO immunoreactivity was found especially in lung epithelial cells (Hayashi et al., 2004; Lee et al., 2017). Furthermore, published data in the context of other murine infections have shown similar mIDO expression. mIDO protein and/or mRNA was found in the lungs of mice experimentally infected with the influenza A virus (Gaelings et al., 2017), the pathogenic fungus *Paracoccidioides brasiliensis* (Araújo et al., 2014), and the pathogenic bacterium *Mycobacterium tuberculosis* (*M. tuberculosis*) (Blumenthal et al., 2012), thus indicating that mIDO does function as an antimicrobial effector mechanism in murine lungs *in vivo*.

During *T. gondii* infection, we found reduced tryptophan concentrations in sera of WT animals, which were even more pronounced in lung tissue, confirming previously published data (Silva et al., 2002; Murakami et al., 2012). The same samples were tested for their kynurenine concentration, as

kynurenine is a degradation product of tryptophan. The decreased tryptophan concentrations were accompanied with an increase in kynurenine concentrations. However, we also detected a drop in tryptophan concentrations in infected IDO$^{-/-}$ mice. This observation is unlikely due to possible mIDO2 activity, since we did not detect mIDO2 mRNA in the majority of samples. Furthermore, the tryptophan drop in the IDO$^{-/-}$ mice is not accompanied by an increase of kynurenine. Therefore, we suggest that enhanced protein biosynthesis by host cells and the rapidly proliferating *T. gondii* tachyzoites during the acute phase of toxoplasmosis are responsible for the decreased tryptophan concentration in the serum of infected animals. Our finding supports this hypothesis, since there is no evidence for an enhanced tryptophan cleavage in the lungs of infected, IDO$^{-/-}$ mice.

We can confirm the observation by Divanovic et al. that IDO$^{-/-}$ mice show no phenotype compared to the WT during acute toxoplasmosis, but rather behave similarly (data not shown). Furthermore, they reported that treatment of chronically infected WT mice with the IDO inhibitor 1-methyl-D-tryptophan (1-D-MT) resulted in *T. gondii* encephalitis (Divanovic et al., 2012). In a previous publication, Murakami et al. reported reduced mRNA expression of the *T. gondii* surface antigen 2 in lungs of *T. gondii*-infected IDO$^{-/-}$ mice compared to WT mice 7 dpi, indicating a lower parasite load or a reduced metabolic activity. Therefore, we analyzed the parasite load in the lungs of the infected animals by detecting a *T. gondii*-specific DNA sequence. Here, we did not detect a significant difference in the *T. gondii* load in lungs of WT or IDO$^{-/-}$ mice. Again, a possible involvement of mIDO2 to compensate for the lack of mIDO1 is unlikely, since mIDO2 mRNA was rare and detectable only at low levels in infected IDO$^{-/-}$ and WT mice. Another tryptophan-degrading enzyme—tryptophan 2,3-dioxygenase (TDO)—might, however, be involved. Human TDO has been described by us to mediate antimicrobial and immunoregulatory effects similar to human IDO (Schmidt et al., 2009). Human TDO has been identified by Hsu et al. (2016) as the main tryptophan-degrading enzyme in human lung cancer-associated fibroblasts. Due to these findings, we have recently established a mIDO1 and mTDO double-deficient mouse strain to further elucidate the involvement of mIDO1 and mTDO during murine infections.

We have shown that a tryptophan concentration of <1 μg/ml is necessary to inhibit bacterial (*S. aureus*) growth as well as human T cell proliferation *in vitro* (Müller et al., 2009). Despite our current finding that the tryptophan concentration in murine lung tissue is strongly reduced during *T. gondii* infection, we could not detect increased parasite loads in lungs of IDO$^{-/-}$ animals, even though *T. gondii* is tryptophan auxotroph. Thus, the tryptophan depletion 7 dpi might not be sufficient to mediate antiparasitic effects *in vivo*. Detailed information concerning the minimal tryptophan concentration for *T. gondii* growth *in vivo* is not available. Our data clearly showed a time-dependent increase of miNOS in lung tissue of infected WT mice on transcript level but could not detect a difference between WT and IDO$^{-/-}$ mice 7 dpi. However, in lungs of IDO$^{-/-}$ mice, we could detect miNOS protein earlier post-infection compared to the WT. Thus, iNOS expression in murine tissues might mediate parasite control during acute toxoplasmosis. This might be another reason why we did not find mIDO1 to be involved in the control of the rapidly replicating tachyzoites during acute toxoplasmosis. iNOS is a previously described antimicrobial defense mechanism and is induced in *T. gondii*-infected mice (Khan et al., 1997). However, mice deficient for iNOS showed prolonged survival in comparison to WT mice (Khan et al., 1997). Detailed analyses showed that enhanced liver degeneration, extensive ulceration, and necrosis in the small intestine were responsible for the earlier death of iNOS-expressing WT mice (Khan et al., 1997). We found higher nitrite accumulation in supernatants of *ex vivo* splenocyte cultures from infected IDO$^{-/-}$ mice compared to infected WT mice. Ye et al. have reported a similar observation in a stem cell transplantation model. They showed that 1-methyl-DL-tryptophan mediated inhibition of mIDO resulted in increased NO concentration in the supernatant of mixed lymphocyte cultures with lymphocytes isolated from BALB/c and C57BL/6 mice (Ye et al., 2017). This indicates, on the one hand, that IDO is influencing NO production. On the other hand, we (Däubener et al., 1999) and others (Bando et al., 2018) found that iNOS can block IDO-mediated antimicrobial effects. Thus, we suggest that mIDO1 and iNOS interact during acute toxoplasmosis and that mIDO1 activity might be required for the regulation of iNOS activity during acute toxoplasmosis in WT mice. Higher iNOS activity might compensate for the missing mIDO1 in IDO$^{-/-}$ mice, whereby potential antiparasitic effects of mIDO1 were not detectable in our experimental setup. The herein mentioned detection of miNOS protein supports this suggestion. Thus, infection experiments with mice deficient for mIDO1 and iNOS might be of interest, since Scharton-Kersten et al. (1997) reported that iNOS-deficient mice can survive acute toxoplasmosis and control parasite growth at the site of infection *via* NO-independent mechanisms. This observation might be due to other aforementioned defense mechanisms (e.g., GTPases) or due to IDO activity. However, that remains to be shown.

Experimental evidence that IDO mediates antimicrobial effects directly *via* tryptophan depletion in mice came from *in vivo* experiments with bacterial infections. For example, Peng and Monack published that genes in the tryptophan biosynthesis pathway are essential for the pathogenic bacterium *Francisella novicida* (*F. novicida*) to multiply in lungs of C57BL/6 mice (Peng and Monack, 2010). Thereafter, bacteria deficient in tryptophan synthesis were constructed, and it was found that this strain had lost its capacity to replicate in the lungs of C57BL/6 mice. In lungs of IDO$^{-/-}$ mice, this tryptophan auxotrophic *F. novicida* strain was able to replicate, thus suggesting that tryptophan depletion *via* mIDO1 did protect the WT mice from the bacterial infection. Comparable data were obtained with a pharmacologic blockage of tryptophan synthesis in *M. tuberculosis*. Zhang et al. showed that a blockage of tryptophan synthesis by halogenated anthranilate analogs disrupted tryptophan biosynthesis in *M. tuberculosis*. Treatment of infected mice with this compound resulted in an inhibition of bacterial growth (Zhang et al., 2013). Inhibition of IDO in macaques during experimental infections with *M. tuberculosis* led to reduced bacterial burden, indicating a better control of the *M. tuberculosis* infection in treated animals

(Gautam et al., 2018). However, Gautam et al. used 1-D-MT, which is not an IDO inhibitor but is rather described to inhibit IDO-mediated immunoregulatory functions (Metz et al., 2012). Therefore, the observed effects in macaques might be due to an enhanced immune reaction against *M. tuberculosis*.

In mice, mIDO expressing plasmacytoid DCs have been reported to suppress T cell responses in tumor-draining lymph nodes (Munn et al., 2004). Furthermore, DCs that express IDO have been linked to several other immunoregulatory functions, for example, the differentiation of regulatory T cells (Grohmann et al., 2017). In addition, tolerance toward self-antigens is regulated by mIDO in the marginal zones of the murine spleen (Ravishankar et al., 2012). Therefore, it was of interest to analyze whether mIDO1 is involved in the T cell suppression, seen during an acute *T. gondii*-infected mouse.

We measured T cell responses from *in vitro* mitogen-stimulated splenocytes, isolated from *T. gondii*-infected mice. Here, we observed a strong suppression of the T cell proliferation in splenocytes from infected compared to naïve mice. However, there was no difference between $IDO^{-/-}$ and WT mice in our experiments, indicating that mIDO1 is not a major factor that regulates the observed T cell suppression. Previous experiments by Chan et al. (1986) have indicated that IL-2 availability as well as macrophages (as potential NO producers) are involved in the T cell suppression observed during acute toxoplasmosis.

Supplementation of IL-2 alone did not influence the proliferation of T cells in our setup, as reported by Khan and coworkers. They observed an increase in the T cell proliferation upon *in vitro* supplementation of IL-2 during mitogen stimulation of purified $CD4^+$ T cells (Khan et al., 1996). In our setup, we stimulated splenocytes consisting not only of T cells but rather of a broad variety of cell types, including macrophages. T cell proliferation has also been reported to be influenced by NO derived from activated macrophage before (Albina et al., 1991; Patton et al., 2002). Inhibition of NOS in our experiments increased the proliferation of T cells derived from both infected mouse strains significantly. IL-2 supplementation and NOS inhibition in combination further increased T cell proliferation, but it did not reach the proliferation level of naïve T cells.

Salinas et al. (2014) have demonstrated that conventional T cells compete with regulatory T cells for available IL-2 in purified T cells isolated during acute toxoplasmosis, induced by infection of C57BL/6 mice orally with 50 *T. gondii* ME49 cysts. With our finding that splenocytes from $IDO^{-/-}$ mice behave like splenocytes from WT mice during *ex vivo* mitogen stimulation, we suggest that the T cell suppression during acute toxoplasmosis is mediated by NOS activity and might even be mediated by IL-2 deprivation as described by Salinas et al. (2014). However,in that case, induction of regulatory T cells would then be independent of mIDO1 in the described T cell suppression during acute toxoplasmosis.

ETHICS STATEMENT

This study was performed in strict compliance with the German Animal Welfare Act. The experiments were authorized by the North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection (Permit# 84 02.04.2013.A271, 84 02.04.2013.A495 and 84 02.04.2016.A508). All efforts were made to minimize animal suffering during the experiments.

AUTHOR CONTRIBUTIONS

WD and IF conceived and supervised the study. C-MU, AD, and WD designed the experiments, prepared the figures, and wrote the manuscript. C-MU and AD performed the majority of experiments and analyzed the data. TB, AT, SC, SE, and KS-B performed the experiments. US supervised animal experiments. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00184/full#supplementary-material

REFERENCES

Adams, L. B., Hibbs, J. B., 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.

Albina, J. E., Abate, J. A., and Henry, W. L. Jr. (1991). Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation. Role of IFN-gamma in the induction of the nitric oxide-synthesizing pathway. *J. Immunol.* 147, 144–148.

Araújo, E. F., Loures, F. V., Bazan, S. B., Feriotti, C., Pina, A., Schanoski, A. S., et al. (2014). Indoleamine 2,3-dioxygenase controls fungal loads and immunity in paracoccidioidomicosis but is more important to susceptible than resistant hosts. *PLoS Negl. Trop. Dis.* 8:e3330. doi: 10.1371/journal.pntd.0003330

Bando, H., Lee, Y., Sakaguchi, N., Pradipta, A., Ma, J. S., Tanaka, S., et al. (2018). Inducible nitric oxide synthase is a key host factor for toxoplasma GRA15-dependent disruption of the gamma interferon-induced antiparasitic human response. *MBio* 9:e01738-18. doi: 10.1128/mBio.01738-18

Bekpen, C., Hunn, J. P., Rohde, C., Parvanova, I., Guethlein, L., Dunn, D. M., et al. (2005). The interferon-inducible p47 (IRG) GTPases in vertebrates: loss

of the cell autonomous resistance mechanism in the human lineage. *Genome Biol.* 6:R92. doi: 10.1186/gb-2005-6-11-r92

Blumenthal, A., Nagalingam, G., Huch, J. H., Walker, L., Guillemin, G. J., Smythe, G. A., et al. (2012). *M. tuberculosis* induces potent activation of IDO-1, but this is not essential for the immunological control of infection. *PLoS ONE* 7:e37314. doi: 10.1371/journal.pone.0037314

Burg, J. L., Grover, C. M., Pouletty, P., and Boothroyd, J. C. (1989). Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *J. Clin. Microbiol.* 27, 1787–1792.

Chan, J., Siegel, J. P., and Luft, B. J. (1986). Demonstration of T-cell dysfunction during acute toxoplasma infection. *Cell. Immunol.* 98, 422–433. doi: 10.1016/0008-8749(86)90301-1

Däubener, W., Posdziech, V., Hadding, U., and MacKenzie, C. R. (1999). Inducible parasitic effector mechanisms in human uroepithelial cells: tryptophan degradation vs. NO production. *Med. Microbiol. Immunol.* 187, 143–147. doi: 10.1007/s004300050086

Däubener, W., Schmidt, S. K., Heseler, K., Spekker, K. H., and MacKenzie, C. R. (2009). Antimicrobial and immunoregulatory effector mechanisms in human endothelial cells indoleamine 2,3-dioxygenase versus inducible nitric oxide synthase. *Thromb. Haemost.* 102, 1110–1116. doi: 10.1160/TH09-04-0250

Degrandi, D., Kravets, E., Konermann, C., Beuter-Gunia, C., Klümpers, V., Lahme, S., et al. (2013). Murine guanylate binding protein 2 (mGBP2) controls *Toxoplasma gondii* replication. *Proc. Natl. Acad. Sci. U.S.A.* 110, 294–299. doi: 10.1073/pnas.1205635110

Dimier, I. H., and Bout, D. T. (1998). Interferon-γ-activated primary enterocytes inhibit *Toxoplasma gondii* replication: a role for intracellular iron. *Immunology* 94, 488–495. doi: 10.1046/j.1365-2567.1998.00553.x

Ding, A. H., Nathan, C. F., and Stuehr, D. J. (1988). Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immunol.* 141, 2407–2412.

Divanovic, S., Sawtell, N. M., Trompette, A., Warning, J. I., Dias, A., Cooper, A. M., et al. (2012). Opposing biological functions of tryptophan catabolizing enzymes during intracellular infection. *J. Infect. Dis.* 205, 152–161. doi: 10.1093/infdis/jir621

Dubey, J. P., and Frenkel, J. K. (1998). Toxoplasmosis of rats: a review, with considerations of their value as an animal model and their possible role in epidemiology. *Vet. Parasitol.* 77, 1–32. doi: 10.1016/S0304-4017(97)00227-6

Gaelings, L., Söderholm, S., Bugai, A., Fu, Y., Nandania, J., Schepens, B., et al. (2017). Regulation of kynurenine biosynthesis during influenza virus infection. *FEBS J.* 284, 222–236. doi: 10.1111/febs.13966

Gautam, U. S., Foreman, T. W., Bucsan, A. N., Veatch, A. V., Alvarez, X., Adekambi, T., et al. (2018). *In vivo* inhibition of tryptophan catabolism reorganizes the tuberculoma and augments immune-mediated control of Mycobacterium tuberculosis. *Proc. Natl. Acad. Sci. U.S.A.* 115, E62–E71. doi: 10.1073/pnas.1711373114

Gazzinelli, R. T., Mendonça-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. doi: 10.1016/j.chom.2014.01.004

Grohmann, U., Mondanelli, G., Belladonna, M. L., Orabona, C., Pallotta, M. T., Iacono, A., et al. (2017). Amino-acid sensing and degrading pathways in immune regulation. *Cytokine Growth Factor Rev.* 35, 37–45. doi: 10.1016/j.cytogfr.2017.05.004

Hayashi, T., Beck, L., Rossetto, C., Gong, X., Takikawa, O., Takabayashi, K., et al. (2004). Inhibition of experimental asthma by indoleamine 2,3-dioxygenase. *J. Clin. Invest.* 114, 270–279. doi: 10.1172/JCI21275

Hsu, Y. L., Hung, J. Y., Chiang, S. Y., Jian, S.-F., Wu, C. Y., Lin, Y. S., et al. (2016). Lung cancer-derived galectin-1 contributes to cancer associated fibroblast-mediated cancer progression and immune suppression through TDO2/kynurenine axis. *Oncotarget* 7, 27584–27598. doi: 10.18632/oncotarget.8488

Hunn, J. P., Feng, C. G., Sher, A., and Howard, J. C. (2011). The immunity-related GTPases in mammals: a fast-evolving cell-autonomous resistance system against intracellular pathogens. *Mamm. Genome* 22, 43–54. doi: 10.1007/s00335-010-9293-3

Johnston, A. C., Piro, A., Clough, B., Siew, M., Virreira Winter, S., Coers, J., et al. (2016). Human GBP1 does not localize to pathogen vacuoles but restricts *Toxoplasma gondii*. *Cell. Microbiol.* 18, 1056–1064. doi: 10.1111/cmi.12579

Khan, I. A., Matsuura, T., and Kasper, L. H. (1996). Activation-mediated CD4+T cell unresponsiveness during acute *Toxoplasma gondii* infection in mice. *Int. Immunol.* 8, 887–896. doi: 10.1093/intimm/8.6.887

Khan, I. A., Schwartzman, J. D., Matsuura, T., and Kasper, L. H. (1997). A dichotomous role for nitric oxide during acute *Toxoplasma gondii* infection in mice. *PNAS* 94, 13955–13960. doi: 10.1073/pnas.94.25.13955

Krishnamurthy, S., Konstantinou, E. K., Young, L. H., Gold, D. A., and Saeij, J. P. J. (2017). The human immune response to Toxoplasma: autophagy versus cell death. *PLoS Pathog.* 13:e1006176. doi: 10.1371/journal.ppat.1006176

Lee, S. M., Park, H. Y., Suh, Y. S., Yoon, E. H., Kim, J., Jang, W. H., et al. (2017). Inhibition of acute lethal pulmonary inflammation by the IDO–AhR pathway. *Proc. Natl. Acad. Sci. U.S.A.* 114, E5881–E5890. doi: 10.1073/pnas.1615280114

Löb, S., Königsrainer, A., Rammensee, H. G., Opelz, G., and Terness, P. (2009). Inhibitors of indoleamine-2,3-dioxygenase for cancer therapy: can we see the wood for the trees? *Nat. Rev. Cancer* 9, 445–452. doi: 10.1038/nrc2639

MacKenzie, C., Heseler, K., Müller, A., and Däubener, W. (2007). Role of indoleamine 2,3-dioxygenase in antimicrobial defence and immunoregulation: tryptophan depletion versus production of toxic kynurenines. *Curr. Drug Metab.* 8, 237–244. doi: 10.2174/138920007780362518

Meisel, R., Brockers, S., Heseler, K., Degistirici, O., Bülle, H., Woite, C., et al. (2011). Human but not murine multipotent mesenchymal stromal cells exhibit broad-spectrum antimicrobial effector function mediated by indoleamine 2,3-dioxygenase. *Leukemia* 25, 648–654. doi: 10.1038/leu.2010.310

Metz, R., Rust, S., DuHadaway, J. B., Mautino, M. R., Munn, D. H., Vahanian, N. N., et al. (2012). IDO inhibits a tryptophan sufficiency signal that stimulates mTOR: a novel IDO effector pathway targeted by D-1-methyl-tryptophan. *Oncoimmunology* 1, 1460–1468. doi: 10.4161/onci.21716

Müller, A., Heseler, K., Schmidt, S. K., Spekker, K., MacKenzie, C. R., and Däubener, W. (2009). The missing link between indoleamine 2,3-dioxygenase mediated antibacterial and immunoregulatory effects. *J. Cell. Mol. Med.* 13, 1125–1135. doi: 10.1111/j.1582-4934.2008.00542.x

Munn, D. H., Shafizadeh, E., Attwood, J. T., Bondarev, I., Pashine, A., and Mellor, A. L. (1999). Inhibition of T cell proliferation by macrophage tryptophan catabolism. *J. Exp. Med.* 189, 1363–1372. doi: 10.1084/jem.189.9.1363

Munn, D. H., Sharma, M. D., Hou, D., Baban, B., Lee, J. R., Antonia, S. J., et al. (2004). Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. *J. Clin. Invest.* 114, 280–290. doi: 10.1172/JCI21583

Munn, D. H., Zhou, M., Attwood, J. T., Bondarev, I., Conway, S. J., Marshall, B., et al. (1998). Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science* 281, 1191–1193. doi: 10.1126/science.281.5380.1191

Murakami, Y., Hoshi, M., Hara, A., Takemura, M., Arioka, Y., Yamamoto, Y., et al. (2012). Inhibition of increased indoleamine 2,3-dioxygenase activity attenuates *Toxoplasma gondii* replication in the lung during acute infection. *Cytokine* 59, 245–251. doi: 10.1016/j.cyto.2012.04.022

Patton, E. A., La Flamme, A. C., Pedras-Vasoncelos, J. A., and Pearce, E. J. (2002). Central role for interleukin-4 in regulating nitric oxide-mediated inhibition of T-cell proliferation and gamma interferon production in schistosomiasis. *Infect. Immun.* 70, 177–184. doi: 10.1128/IAI.70.1.177-184.2002

Pelloux, H., Guy, E., Angelici, M. C., Aspöck, H., Bessières, M. H., Blatz, R., et al. (1998). A second European collaborative study on polymerase chain reaction for *Toxoplasma gondii*, involving 15 teams. *FEMS Microbiol. Lett.* 165, 231–237. doi: 10.1111/j.1574-6968.1998.tb13151.x

Peng, K., and Monack, D. M. (2010). Indoleamine 2,3-dioxygenase 1 is a lung-specific innate immune defense mechanism that inhibits growth of *Francisella tularensis* tryptophan auxotrophs. *Infect. Immun.* 78, 2723–2733. doi: 10.1128/IAI.00008-10

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. doi: 10.1073/pnas.81.3.908

Ravishankar, B., Liu, H., Shinde, R., Chandler, P., Baban, B., Tanaka, M., et al. (2012). Tolerance to apoptotic cells is regulated by indoleamine 2,3-dioxygenase. *Proc. Natl. Acad. Sci. U.S.A.* 109, 3909–3914. doi: 10.1073/pnas.1117736109

Salinas, N., Olguín, J. E., Castellanos, C., and Saavedra, R. (2014). T cell suppression *in vitro* during *Toxoplasma gondii* infection is the result of IL-2 competition between tregs and T cells leading to death of proliferating T cells. *Scand. J. Immunol.* 79, 1–11. doi: 10.1111/sji.12120

Sasai, M., Pradipta, A., and Yamamoto, M. (2018). Host immune responses to *Toxoplasma gondii*. *Int. Immunol.* 30, 113–119. doi: 10.1093/intimm/dxy004

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, 1261–1274. doi: 10.1084/jem.185.7.1261

Schlüter, D., Däubener, W., Schares, G., Groß, U., Pleyer, U., and Lüder, C. (2014). Animals are key to human toxoplasmosis. *Int. J. Med. Microbiol.* 304, 917–929. doi: 10.1016/j.ijmm.2014.09.002

Schmidt, S. K., Müller, A., Heseler, K., Woite, C., Spekker, K., MacKenzie, C. R., et al. (2009). Antimicrobial and immunoregulatory properties of human tryptophan 2,3-dioxygenase. *Eur. J. Immunol.* 39, 2755–2764. doi: 10.1002/eji.200939535

Schwartzman, J. D., Gonias, S. L., and Pfefferkorn, E. R. (1990). Murine gamma interferon fails to inhibit *Toxoplasma gondii* growth in murine fibroblasts. *Infect. Immun.* 58, 833–834.

Silva, N. M., Rodrigues, C. V., Santoro, M. M., Reis, L. F., Alvarez-leite, J. I., Gazzinelli R., T. et al. (2002). Expression of indoleamine and kynurenine formation during *in vivo* infection with *Toxoplasma gondii*: induction by endogenous gamma interferon and requirement of interferon regulatory factor 1 expression of indoleamine 2,3-dioxygenase, tryptophan. *Infect. Immun.* 70, 859–868. doi: 10.1128/IAI.70.2.859-868.2002

Spekker-Bosker, K., Ufermann, C.-M., Maywald, M., Zimmermann, A., Domröse, A., Woite, C., et al. (2019). hCMV-mediated immune escape mechanisms favor pathogen growth and disturb the immune privilege of the eye. *Int. J. Mol. Sci.* 20, 1–13. doi: 10.3390/ijms20040858

Wilking, H., Thamm, M., Stark, K., Aebischer, T., and Seeber, F. (2016). Prevalence, incidence estimations, and risk factors of *Toxoplasma gondii* infection in Germany: a representative, cross-sectional, serological study. *Sci. Rep.* 6, 1–9. doi: 10.1038/srep22551

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

Ye, Q. X., Xu, L. H., Shi, P. J., Xia, T., and Fang, J. P. (2017). Indoleamine 2,3-dioxygenase and inducible nitric oxide synthase mediate immune tolerance induced by CTLA4Ig and anti-CD154 hematopoietic stem cell transplantation in a sensitized mouse model. *Exp. Ther. Med.* 14, 1884–1891. doi: 10.3892/etm.2017.4722

Yeung, A. W., Terentis, A. C., King, N. J., and Thomas, S. R. (2015). Role of indoleamine 2,3-dioxygenase in health and disease. *Clin. Sci.* 129, 601–672. doi: 10.1042/CS20140392

Zhang, Y. J., Reddy, M. C., Ioerger, T. R., Rothchild, A. C., Dartois, V., Schuster, B. M., et al. (2013). Tryptophan biosynthesis protects mycobacteria from CD4 T-cell-mediated killing. *Cell* 155, 1296–1308. doi: 10.1016/j.cell.2013.10.045

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Supplementary Material

Indoleamine 2,3-Dioxygenase Activity During Acute Toxoplasmosis and the Suppressed T Cell Proliferation in Mice

Christoph-Martin Ufermann[1†], Andreas Domröse[1†], Timo Babel[1], Anne Tersteegen[1], Sevgi Can Cengiz[2], Silvia Kathrin Eller[1], Katrin Spekker-Bosker[1], Ursula Regina Sorg[1], Irmgard Förster[2], Walter Däubener[1*]

[1] Institute of Medical Microbiology and Hospital Hygiene, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany
[2] Immunology and Environment, Life & Medical Sciences (LIMES) Institute, University of Bonn, Bonn, Germany.

[†]These authors have contributed equally to this work

***Correspondence:**

Prof. Dr. Walter Däubener
Institute of Medical Microbiology and Hospital Hygiene
Heinrich-Heine-University Düsseldorf
Universitätsstr. 1, Bldg. 22.21
40225 Düsseldorf, Germany
Phone: +49 (0) 211-81-12464
Fax: +49 (0) 211-81-15906
e-mail: daeubene@uni-duesseldorf.de

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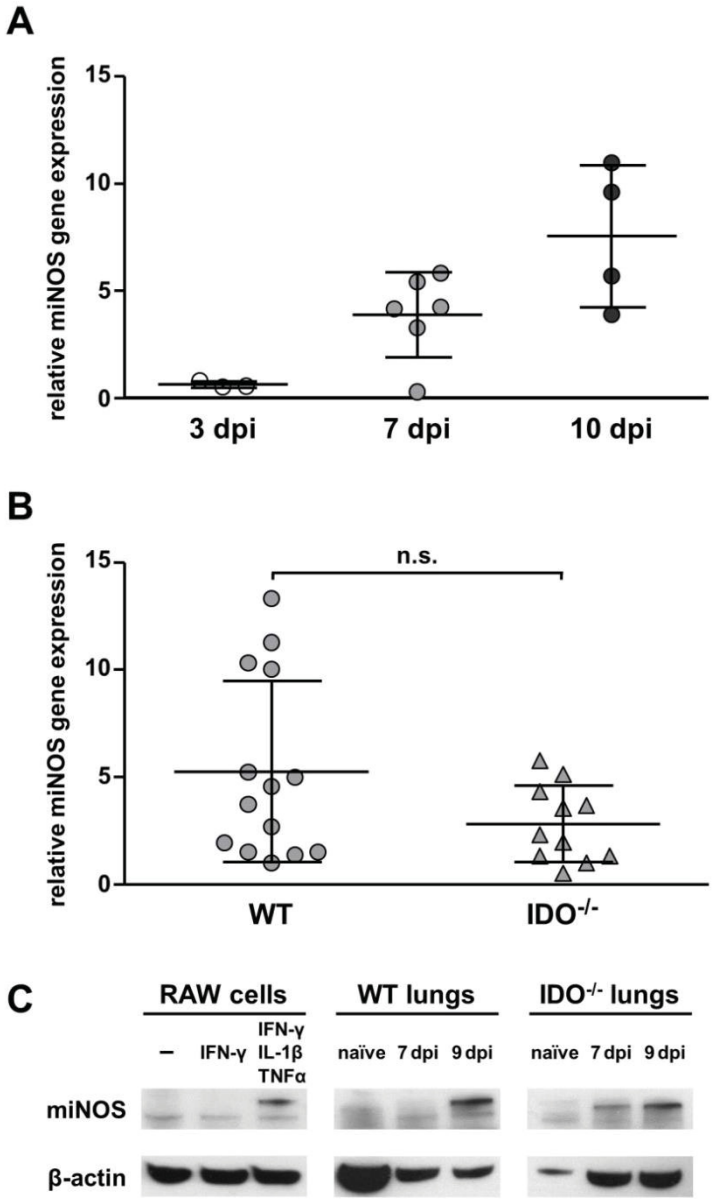
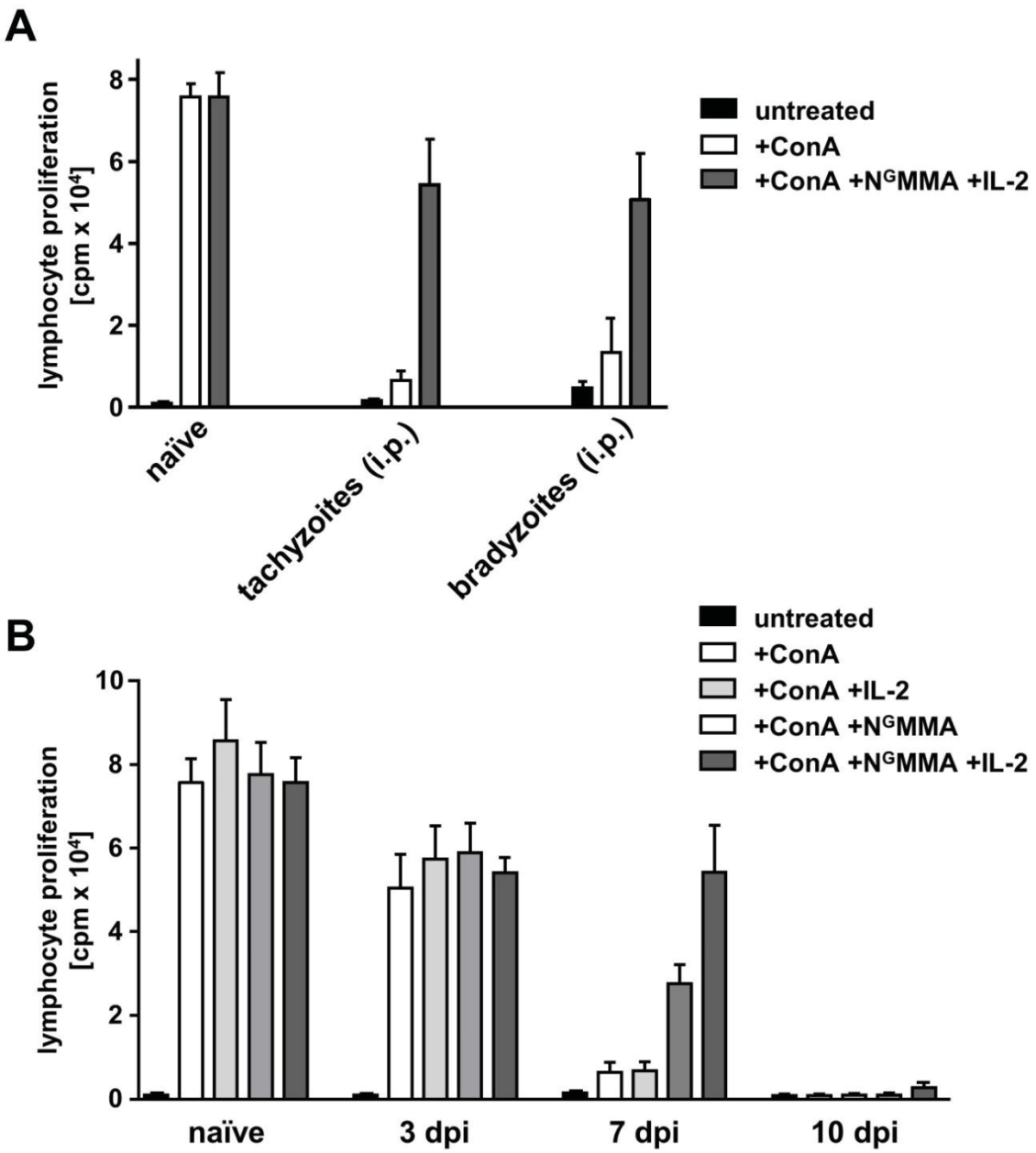
Supplementary Table S1 Oligonucleotides used in this study.

Gene expression		
Primer Name	**Primer Sequence [5'->3']**	**Roche Probe ID**
mβ-actin-fw	TGACAGGATGCAGAAGGAGA	106
mβ-actin-rv	CGCTCAGGAGGAGCAATG	
mGBP2-fw	TGAGTACCTGGAACATTCACTGAC	17
mGBP2-rv	AGTCGCGGCTCATTAAAGC	
mIDO1-fw	GGGCTTCTTCCTCGTCTCTC	2
mIDO1-rv	TGGATACAGTGGGGATTGCT	
mIDO2-fw	GTCCTTGGGGAGATACCACA	12
mIDO2-rv	CCAAGGCTTGTAATGATCTGG	
miNOS-fw	CTTTGCCACGGACGAGAC	13
miNOS-rv	TGTACTCTGAGGGCTGACACA	
Parasite load		
Primer Name	**Primer Sequence [5'->3']**	
*Tg*B1-fw	GCTAAAGGCGTCATTGCTGTT	
*Tg*B1-rv	GGCGGAACCAACGGAAAT	
*Tg*B1-probe	FAM-ATCGCAACGGAGTTCTTCCCAGACGT-BHQ1	



Supplementary Figure S1 Expression of miNOS in murine lung tissue during *Toxoplasma gondii* infection. Gene and protein expression of murine inducible nitric oxide synthase (miNOS) in tissues from naïve or *T. gondii* ME49 infected wild-type (WT) and indoleamine 2,3-dioxygenase 1-deficient ($IDO^{-/-}$) mice at different time points post infection. Expression of miNOS in lung tissue homogenates of infected mice relative to their expression in naïve control samples on 3, 7 and 10 days post infection (dpi) **(A)**. Expression of miNOS in lung tissue homogenates of infected WT and $IDO^{-/-}$ mice relative to their expression in naïve control samples at day 7 post infection (dpi) **(B)**. Western blot analysis shows miNOS and β-actin protein in lung tissue of naïve and infected WT and $IDO^{-/-}$ mice as well as untreated (-) and stimulated (IFN-γ 100 U/mL or IFN-γ 100 U/mL + IL-1β 100 U/mL + TNFα 100 U/mL) RAW 264.7 cells as negative and positive controls respectively **(C).** IFN-γ, IL-1β, TNFα were purchased from R&D Systems (Minneapolis, USA). qPCR data were normalized to the housekeeping gene β-actin and were represented as $2^{-\Delta\Delta CT}$ (naïve vs. infected) in scattered dot plots and means ± standard deviation. The Student´s *t*-test (unpaired, two-tailed) was used to determine statistical differences marked with asterisks (n.s. = not significant).



Supplementary Figure S2 Mitogen induced lymphocytes proliferation responses in murine splenocytes. Splenocytes were isolated from uninfected (naïve; n = 3) or *T. gondii* ME49 tachyzoite (intraperitoneal (i.p.) dose: 10^5 tachyzoites; n = 3) or bradyzoite (i.p. dose: 20 lysed cysts; n = 3) infected C57BL/6 wild-type (WT) mice at 7 days post infection (dpi) **(A)**. Splenocytes were isolated from uninfected (naïve; n = 3) or *T. gondii* ME49 tachyzoite infected (i.p. dose: 10^5 tachyzoites) WT mice at 3 dpi (n = 3), 7 dpi (n = 3) and 10 dpi (n = 3) **(B)**. Splenic T cell cultures were stimulated with the mitogen concanavalin A (ConA, 1 µg/mL) *ex vivo*. Additional supplementation of recombinant interleukin-2 (IL-2, 5 ng/mL) and the nitric oxide synthase (NOS) inhibitor N^G-monomethyl-L-arginine (N^GMMA, 100 µg/mL) was done as indicated. Lymphocyte proliferation was determined with the ^{3}H-thymidine method. Data were represented as means of triplicate measurements + standard error of the mean of one experiment.

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4. Discussion and concluding remarks

The results obtained in this thesis revealed a profoundly disordered immune response of LTβR$^{-/-}$ mice to an infection with *T. gondii*, resulting in an impaired T cell response, dysregulated cytokine patterns, dysregulated IFNγ driven anti-parasitic responses, and largely abrogated immunoglobulin class switching. Ultimately, due to all these discovered defects, LTβR$^{-/-}$ mice are not able to control the parasite and succumb to a *T. gondii* infection.

To investigate the cause of infection in LTβR$^{-/-}$ mice, the parasite burden was analyzed. Not unexpectedly, more parasites were detected in lung, spleen and muscle tissue of LTβR$^{-/-}$ mice, which can be explained by the immune defects uncovered in this thesis. Interestingly though, LTβR$^{-/-}$ animals had fewer parasites in the brain. The trigger for the migration of infectious tachyzoites into the brain is not completely understood, but it is known that under immune pressure of the host immune system tachyzoites convert to metabolically less active bradyzoites which form tissue cysts and establish a chronic infection (Lyons, McLeod et al. 2002). Two explanations for the reduced cyst numbers in the brain of LTβR$^{-/-}$ mice are possible. Parasites might not be driven into the chronic stage of infection because of the lack of efficient immune pressure. Or, more likely, reduced parasite numbers are caused by the lack of specific cell populations, especially DCs and macrophages that are used by the parasite as a "Trojan horse" to shuttle into the brain (Courret, Darche et al. 2006, Lambert, Hitziger et al. 2006, Lambert, Vutova et al. 2009). In addition, the blood-brain barrier consists of endothelial cells. Since the LTβR is expressed on endothelial cells and has been shown to have an essential role in attracting $LT\alpha_1\beta_2$ expressing (T) cells to specific locations (e.g. LTi cells in the lymph nodes) during the development of secondary lymphoid organs (Scandella, Bolinger et al. 2008), it might be postulated that in LTβR$^{-/-}$ mice specific immune cells cannot cross into the brain tissue.

One overt symptom of acute *T. gondii* infection also seen in patients with severe toxoplasmosis is increased spleen size. While increased spleen size/weight during infection was observed in both cohorts, WT mice showed a stronger increase which is at least partially due to inflammatory swelling as LTβR$^{-/-}$ spleens actually contained increased splenocytes numbers compared to WT animals. At the same time, LTβR$^{-/-}$ mice show reduced numbers of DCs, macrophages, eosinophils, neutrophils and inflammatory monocytes compared to WT mice. This difference between increased cellularity and reduced numbers of specific cell populations could be due to the presence/absence of other subpopulations so far not analyzed, but initial observations strongly suggest that LTβR$^{-/-}$ mice have markedly increased numbers of apoptotic cells. Further analysis of splenocytes is necessary to clarify the nature of the increased cellularity in spleens of infected LTβR$^{-/-}$ animals.

While *T. gondii* specific $CD8^+$ T cells differentiate in $LT\beta R^{-/-}$ mice in comparable numbers to WT mice, their functionality is markedly decreased. $LT\beta R^{-/-}$ mice have significantly lower numbers of *T. gondii* specific T cells which contain granzyme B and perforin and appear virtually unable to degranulate. This implies that cytotoxic T cell mediated killing is severely impaired which would contribute to the increased susceptibility of $LT\beta R^{-/-}$ mice. However, the question why *T. gondii* specific $CD8^+$ T cells without the LTβR expand but are not functional remains unsolved.

Since $LT\alpha_1\beta_2$ and LIGHT can both signal via the LTβR (Hehlgans and Pfeffer 2005) the interesting question is which ligand is responsible for the altered cytotoxicity of $CD8^+$ T cells of infected $LT\beta R^{-/-}$ mice. Therefore, bone marrow cells of $LT\beta R^{-/-}$, $LT\alpha^{-/-}$, $LT\beta^{-/-}$ and $LIGHT^{-/-}$ donor mice should be transferred into $Rag^{-/-}\gamma c^{-/-}$ acceptor mice which lack NK-, T-, B cells and ILCs (Sanchez, Cuadra et al. 2013), before infection with *T. gondii.* Immune cell populations should be investigated via flow cytometry as well as the survival of these animals. To further identify cellular subpopulations that might be responsible for the observed phenotype of infected $LT\beta R^{-/-}$ mice, $LT\beta^{-/-}$ and $LIGHT^{-/-}$ acceptor mice should be reconstituted using WT donor $CD4^+$ and $CD8^+$ T cells that were isolated via fluorescence-activated cell sorting (FACS) or magnetic beads.

Interaction of naïve T cells with DCs, important for their activation/maturation, takes place in the lymph nodes which are absent in $LT\beta R^{-/-}$ mice. This may explain why LTβR animals showed less differentiation of naïve $CD4^+$ T cells into $CD4^+$ effector T cells and why they have impaired immunoglobulin (Ig) affinity maturation (Futterer, Mink et al. 1998). Regulatory T cells are reported to use the $LT\alpha_1\beta_2$-LTβR interaction for afferent lymphatic transendothelial migration (Brinkman, Iwami et al. 2016) and to be essential for protection during the acute phase of infection (Tenorio, Olguin et al. 2010). In addition, it has been shown that LTβR blockade induces inflammation and fibrosis associated with decreased regulatory T cell infiltration into grafts in a murine cardiac allograft model (Nakayama and Bromberg 2012). These data suggest that LTβR deficiency might also affect the localization of regulatory T cells *in T. gondii* infected $LT\beta R^{-/-}$ mice, likely contributing to the increased mortality. Flow cytometry of regulatory T cells of *T. gondii* infected WT and $LT\beta R^{-/-}$ is necessary to investigate this hypothesis.

Another approach to investigate the role of the LTβR in regard to various immune cell subpopulations is to use a mouse line that carries a floxed LTβR gene and crossing it with mouse lines carrying a Cre gene under the control of various lineage specific promotors. This would generate mouse lines that are LTβR deficient in various cell lineages. For example Tie2 is an endothelial cell specific promotor, thus an $LT\beta R^{flox/flox}$ x $Tie2ER^{T2}Cre$ line could be used to investigate the importance of LTβR expression on endothelial cells. This mouse line would

be of great value to investigate whether specific immune cells cannot cross the blood-brain barrier in LTβR$^{-/-}$ mice, which could be responsible for the lower parasite numbers in the brain.

It is known that CD8^{+} T cells exhibit perforin dependent anti-cyst activity in the brain of *T. gondii* infected mice (Suzuki 2019). *T. gondii* infected LTβR$^{-/-}$ mice showed reduced numbers of *T. gondii* specific CD8^{+} T cells that are granzyme B and perforin positive so it is likely that a perforin dependent anti-parasitic activity might be already important during the late acute phase of infection in these mice. Despite these observations, the role of cytotoxic T cell mediated killing in *T. gondii* infection remains largely unclear. A protective role for CD4^{+} T cells in the spleens of *T. gondii* infected animals has been shown (Grover, Blanchard et al. 2012), but their role in the brain is largely unexplored. Here, LTβR$^{-/-}$ compared to WT animals showed lower CD4 and CD8 mRNA expression in brain tissue suggesting that these animals might have reduced T cell numbers in the brain. It has been reported that resident and infiltrating T cells in the brain of infected WT animals are essential to control *T. gondii* infection during the chronic phase of infection in the brain (Schluter, Hein et al. 1995, Schaeffer, Han et al. 2009, Wilson, Harris et al. 2009). However, in *T. gondii* infected LTβR$^{-/-}$ animals the role of brain resident and infiltrating immune cells is as yet unexplored. It is unclear whether T cells of *T. gondii* infected LTβR$^{-/-}$ mice are unable to cross the blood-brain barrier as discussed earlier or whether their presence is not (yet) necessary due to the lower parasite burden in the brain of these animals. Further analysis of immune cell populations in brain tissue of *T. gondii* infected LTβR$^{-/-}$ mice is necessary to understand the role of the LTβR with regard to the establishment of chronic toxoplasmosis.

Under specified pathogen-free (SPF) housing conditions LTβR$^{-/-}$ mice do not show an overt phenotype and are obviously able to compensate for the lack of LTβR signaling. However, transcriptomics reveal fundamental differences in gene expression patterns in lung tissue of uninfected LTβR$^{-/-}$ compared to WT mice. In uninfected animals, an increased activation status of LTβR$^{-/-}$ mice was identified and experimental data confirmed increased levels of several pro-inflammatory cytokines in the serum. It is conceivable that the lack of LTβR signaling is compensated by the excess production of other cytokines (e.g. LTβ and TNF) which could in turn induce negative feedback loops creating a precarious balance. However, it might also be possible that LTβR$^{-/-}$ mice already have issues dealing with the commensal flora, due to which they increase cytokine expression. Upon *T. gondii* infection, an immune response is initiated. In WT animals *T. gondii* infection leads to the massive expression of IFNγ and IFNγ induced genes to contain the parasite (Sasai and Yamamoto 2019). Transcriptomic data generated in this study depicts an increased global expression pattern. In infected LTβR$^{-/-}$ mice the up-regulation of various cytokines such as IL-1β, IL-6 and IL-10 reflect a higher activation of the immune response to the infection that is accompanied by upregulation of regulatory molecules

of the IFNγ signaling pathway (e.g. SOCS 3). Ultimately this seems to result in a paralysis of the system as infected LTβR$^{-/-}$ animals show global downregulation of gene expression compared to the uninfected WT controls as well as to the infected WT animals. This could, of course, explain the increased mortality of LTβR$^{-/-}$ mice. Since transcriptome data was generated from lung tissue und cytokine analysis was performed of serum, cytokine expression should also be analyzed in lung tissue for appropriate correlation of these data sets. In addition, different tissues, e.g. spleen or muscle tissue could also be used to generate transcriptome data.

IFNγ signaling is essential for efficient anti-parasitic immune responses since neither IFNγ$^{-/-}$ nor IFNγR$^{-/-}$ mice are able to contain *T. gondii* and consequently succumb early in infection (Deckert-Schluter, Rang et al. 1996, Scharton-Kersten, Wynn et al. 1996). The finding that LTβR$^{-/-}$ compared to WT mice did not upregulate IFNγ in the serum in early stages of infection is concordant with the lack of gene upregulation involved in IFNγ responses found in transcriptome analysis. By day 10 post infection IFNγ levels in WT animals are markedly decreased again. Most probably this reflects parasite containment and transition to the chronic phase of infection while IFNγ expression in LTβR$^{-/-}$ animals is significantly increased since they cannot sufficient control the parasite.

The expression of IFNγ induced effector molecules in lung and brain tissue, such as IDO (Pfefferkorn, Rebhun et al. 1986, Murray, Szuro-Sudol et al. 1989) and iNOS (Scharton-Kersten, Yap et al. 1997, Khan, Matsuura et al. 1998) as well as the upregulation of mGBPs which are essential for an efficient immune response to *T. gondii* (Yamamoto, Okuyama et al. 2012, Degrandi, Kravets et al. 2013, Selleck, Fentress et al. 2013, Behnke, Sorg et al. 2017, Steffens, Beuter-Gunia et al. 2020) is significantly reduced in LTβR$^{-/-}$ animals. IDO and iNOS expression are rather unlikely to solely account for the susceptibility of LTβR$^{-/-}$ mice to *T. gondii*. Ufermann et al. (Ufermann, Domrose et al. 2019) (see 3.3) show that while IDO alone is not sufficient to mediate an anti-parasitic effect *in vivo*, iNOS might mediate parasite containment during acute toxoplasmosis. Mice deficient for iNOS are shown to control parasite replication during acute *T. gondii* infection, but show increased susceptibility during the chronic stage of infection (Scharton-Kersten, Yap et al. 1997). This indicates that IDO and iNOS expression may play only minor roles compared to other effector molecules in regard to parasite containment, at least in mice. Most probably, specifically the expression of mGBPs and IRGs is essential for parasite containment since mice deficient for different IFNγ regulated effector genes (e.g. mGBP2$^{-/-}$, mGBP7$^{-/-}$, IGTP$^{-/-}$ mice) succumb to *T. gondii* infection and show an increase in parasite burden (Taylor, Collazo et al. 2000, Degrandi, Kravets et al. 2013, Steffens, Beuter-Gunia et al. 2020).

The observation that IFNγ expression patterns differ between serum and tissue (lung and brain) of WT and LTβR$^{-/-}$ mice during infection was unexpected. While LTβR$^{-/-}$ and WT mice show comparable IFNγ levels in lung and brain tissue, LTβR$^{-/-}$ mice show a delayed upregulation of protein expression of genes involved in IFNγ signaling, such as (p)STAT1, IRF-1 and mGBPs in both tissues. One explanation could be that while IFNγ is expressed in comparable amounts in lung and brain tissue, it might not be bioavailable in LTβR$^{-/-}$ animals due to sequestration. Therefore, we assume that serum IFNγ is mainly responsible for modulating expression of IFNγ-regulated genes. In the brain, IFNγ expression is not upregulated during the acute of infection in both genotypes, suggesting that up-regulation of effector molecules in WT animals might in this case be dependent on LTβR signaling rather than IFNγ signaling, as LTβR$^{-/-}$ animals show delayed up-regulation of these effector proteins. Moreover, the delayed IFNγ response in sera of LTβR$^{-/-}$ mice is in line with the findings that LTβR$^{-/-}$ mice lack a significant NK cell population (Wu, Sun et al. 2001). This delayed response in LTβR$^{-/-}$ mice could therefore be caused by the absence of the timely IFNγ production by NK cells (Gaddi and Yap 2007) and DCs (Fricke, Mitchell et al. 2006) - especially cDCs and pDCs - in concert with the reduced number of effector and IFNγ producing CD4^{+} T cells (Denkers, Gazzinelli et al. 1993, Gigley 2016). An impaired release of IFNγ by NK cells due to delayed IL-12p40 secretion by DCs during *T. gondii* infection of LTβR$^{-/-}$ mice has been already suggested (Hou, Benson et al. 2011, Behnke, Sorg et al. 2017). However, in this study comparable production of IL-12p70 (consisting of the IL-12p35 and IL-12p40 subunits) in the serum of infected LTβR$^{-/-}$ mice was detected, indicating that the delayed IFNγ response is more likely due to a lack of specific cell populations than to an impaired release via delayed IL-12 production. Besides DCs and NK cells various cell types have been described to produce IFNγ on a single cell level (Scharton-Kersten, Nakajima et al. 1998, Denkers 1999, Darwich, Coma et al. 2009, Wagage, Harms Pritchard et al. 2015, Ivanova, Denton et al. 2019). However, monocytes and macrophages are unlikely to account for a delayed early IFNγ production, since flow cytometry revealed comparable numbers in spleens of WT and LTβR$^{-/-}$ mice (d0 – d7 *p.i.*). ILCs which are also known to produce IFNγ and have been shown to be important in *T. gondii* infection (Dunay and Diefenbach 2018), were not studied in this thesis, but should be investigated in further studies. While IFNγ production was analyzed in serum, lung and brain tissue, immune cell populations were only analyzed in the spleen. In order to appropriately correlate the data, effector functions of immune cells should be investigated in the respective tissues of infected LTβR$^{-/-}$ mice using flow cytometry und functional tests. Moreover, these studies should ideally be performed using a fluorescent *T. gondii* strain because parasite numbers could then easily be measured via flow cytometry. This would also allow to simultaneously determine the parasite burden in tissues and the parasite burden in (tissue) specific immune cell populations. So far, parasite burdens were examined via qRT-PCR

representing total parasite numbers per organ. While immune cell populations differ between WT and LTβR$^{-/-}$ mice it is likely that different cell populations become more or less infected. As LTβR$^{-/-}$ animals show differences in the spleen microarchitecture and lack lymph nodes and Peyer's patches, immune histochemistry analysis could provide valuable information on the localization and distribution of various immune cell populations during the course of infection. For example the migration from splenic naïve CD4^{+} T cell of LTβR$^{-/-}$ mice into other tissues, due to the lack of secondary peripheral organs, could be analyzed via immune histochemistry as discussed above. Also, regarding the fact that LTβR$^{-/-}$ mice studied in this thesis are globally deficient in the LTβR and therefore exhibit altered secondary lymphoid tissue and organ structures (Futterer, Mink et al. 1998), it is advisable to use conditionally LTβR deficient animals for future studies, e.g. inducible Cre mice such as LTβR$^{flox/flox}$ x R26-CreER^{T2} for global knockout of the LTβR. It would be interesting to use cell lineage specific deleter mice to study the role of different immune cell populations (e.g. LTβR$^{flox/flox}$ x LysM-Cre mice for myeloid cells or LTβR$^{flox/flox}$ x CD11c-Cre mice for DCs) during *T. gondii* infection in LTβR$^{-/-}$ mice. This would reveal the role of the LTβR on specific cell linages and ensure that the effects of LTβR signaling on the development of secondary lymphoid tissues and organs would not complicate matters.

The transcriptomics data were also used to generate a pathway analysis to investigate possible interactions between LTβR and IFNγ signaling pathways. This pathway analysis shows a known network of protein interactions and superimposes RNAseq data of uninfected WT and LTβR$^{-/-}$ mice. Notably, the lack of LTβR signaling appeared to slightly increase basal IFNγ expression that could modulate signaling through the IFNγ receptor. It has been reported that changes in IFNγ signaling – even in the absence of pathogens- could in induce a lowgrade pro-inflammatory status via several pathways (Codrici, Albulescu et al. 2018) via several different pathways. Pathway analysis of transcriptomic data from uninfected LTβR$^{-/-}$ mice, as well as mRNA and protein experimental data, generated in this study, are supporting this finding. Other regulatory pathways mediated by IFNγ could also contribute to the general dysregulation of the immune response in LTβR$^{-/-}$ animals. For instance, increased expression of BAFF via PLCγ signaling would influence lymphocyte and especially B cell activation/differentiation. Also, increased ROS production (induced via gp91-phox and p67-phox) could reinforce pro-apoptotic signaling, which may already be enhanced by STAT3 and NFκB mediated signals. These interactions could explain the shift in the various immune cell lineages observed in LTβR$^{-/-}$ mice. However, it has to be kept in mind that a pathway analysis correlates mRNA expression data with published protein interactions. Regulation of translational processes, post-transcriptional and post-translational modifications and other mechanisms that regulate secretion/sequestration of proteins are not taken into account.

This thesis suggests that dysregulation of early, IFNγ dependent signaling pathways is mainly responsible for the increased susceptibility of LTβR$^{-/-}$ mice to *T. gondii* infection and indicates that the lack of LTβR signaling interferes with IFNγ signaling. This idea is supported by the *in vitro* experiments with infected WT and LTβR$^{-/-}$ MEFs that show comparable mGBP expression as well as similar mGBP2 recruitment towards the *T. gondii* PV in both cohorts in the presence of exogenous IFNγ. While the pathway analysis clearly demonstrates that LTβR deficiency could affect IFNγ signaling via multiple pathways, it cannot answer the question of how LTβR signaling interacts with IFNγ signaling on a molecular level. Therefore, IFNγ signaling proteins including phosphorylated variants and inhibitory molecules should be analyzed in infected WT and LTβR$^{-/-}$ mice by Western Blot or mass spectrometry analysis to provide further information. Promising candidates should be further investigated and characterized in respective deficient cell lines or mouse strains. If these are not available promising candidates could be knocked out in cell lines/mice via the CRISPR/Cas9 technique (Yang, Yang et al. 2014, Mout, Ray et al. 2017, Barman, Deb et al. 2020).

LTβR$^{-/-}$ mice lack lymph nodes and are known to have impaired B cell receptor affinity maturation (Futterer, Mink et al. 1998). Immunity towards *T. gondii* infection is described to be T cell mediated and that B cell responses play a significant role in chronic rather than acute infection (Yarovinsky 2014, Dunay and Diefenbach 2018, Sasai, Pradipta et al. 2018). A protective role of B cells in *T. gondii* infection has been described in μMT mice (Chen, Mun et al. 2003) but the authors could not determine whether this effect was due to a lack of parasite specific antibodies. Also, parasite specific antibodies have been described to promote control of *T. gondii* infection in IL-27p28 transgenic mice, but their influence on survival was not clear (Park, DeLong et al. 2019). Here, transcriptomic data was used to generate a host-pathogen network prediction model indicating that the loss of the LTβR affects B cell responses including isotype switching in *T. gondii* infection. This study shows for the first time that LTβR$^{-/-}$ mice are unable to generate *T. gondii* specific IgM in amounts comparable to WT mice and do not produce *T. gondii* specific IgG after infection. It has been reported that infected LTβR$^{-/-}$ mice express reduced amounts of IFNγ which is known to stimulate the isotype class switch to IgG2a (Coffman, Savelkoul et al. 1989). However, the absence of the ability to perform class switching may not only be due to reduced IFNγ production but also to the absence of the required splenic microenvironment, as LTβR$^{-/-}$ mice lack defined splenic germinal centers. In this study the experiments suggest that increased survival of infected LTβR$^{-/-}$ animals depends on the quality of immune serum i.e. the antibody titer. For further analysis a more sensitive method to measure antibody production would be desired to quantify antibody titers. It is therefore of importance to further refine this serum transfer model to address the role of Igs for survival of *T. gondii* infections. This is especially important in view of the fact that in a clinical setting *T. gondii* infection would occur before treatment. It would be interesting to investigate whether

prolonged survival can also be attained when serum transfer (i.e. passive immunization) occurs after the infection. The fact that survival of LTβR$^{-/-}$ animals can be significantly prolonged by transfer of immune serum containing *T. gondii* specific antibodies despite the concurrent impaired T cell functionality emphasizes the major importance of a B cell-mediated immune response in the defence against *T. gondii*, which has not been described so far. LTβR$^{-/-}$ mice that receive immune serum have to be analyzed in more detail in regard to the parasite burden in different organs, the cyst burden in brain tissue as well as overall cytokine levels in serum. Further analysis of these animals is necessary to assess the protective role of antibodies on the one hand and B cell help on the other hand in a *T. gondii* infection. In order to address this, serum transfer and adoptive B cell transfer experiments with immune deficient mice (e.g. RAG$^{-/-}$, lacking mature B and T cells (Mombaerts, Iacomini et al. 1992) and nude mice, lacking T cells (Shire and Pantelouris 1973)) could be performed and analyzed as described above for LTβR$^{-/-}$ mice.

While a robust immune response is induced upon *T. gondii* infection, the parasite has also developed several strategies to evade the immune system. *T. gondii* manipulates host immunity through the control of host gene transcription and dysregulation of signaling pathways that modulate various processes involved in cell adhesion, migration, apoptosis, secretion of immunoregulatory cytokines and production of microbicidal molecules (Lima and Lodoen 2019). In addition, the host-pathogen prediction model indicates that *T. gondii* infection may supress B cell responses in WT mice. This points towards a so far unknown *T. gondii* specific mechanism, most likely a strategy to evade the host immune system. It would be of great interest to further elucidate this mechanism. As a first step, the B cell response to *T. gondii* in WT mice would have to be characterized in detail to identify the stage at which the parasite might interfere with the humoral immune response. Pathway analysis comprising transcriptomic data could help to identify potential regulatory pathways and key molecules that are influenced by *T. gondii*. It could also be helpful to compare the course of *T. gondii* infection with other parasitic infections that elicit effective B cell responses such as *Plasmodium falciparum (Silveira, Dominguez et al. 2018)* or *Neospora caninum* (Teixeira, Marques et al. 2005) infection. Once these pathways and/or molecules are pinpointed, molecular analysis of interaction partners could identify the *T. gondii* effector molecules responsible. Genes that are not upregulated or upregulated inhibitory molecules of *T. gondii* infected WT mice that are involved in B cell responses (transcriptomic data) can then be investigated in more detail to identify a possible *T. gondii* effector gene. These could then be knocked out via the CRISPR/Cas9 technology first in cell lines for verification and characterization and ultimately in mice.

In this thesis a comprehensive characterization of innate and adaptive immune responses of LTβR$^{-/-}$ mice in a *T. gondii* infection model was performed. Figure 8 summarizes and visualizes the main results demonstrating that *T. gondii* infection in LTβR$^{-/-}$ mice results in impaired T cell functionality, dysregulated cytokine patterns, dysregulated IFNγ responses, and absent parasite specific antibodies that lead to an increase in parasite numbers causing the high susceptibility of LTβR$^{-/-}$ mice to *T. gondii* infection. Importantly, survival of infected LTβR$^{-/-}$ mice could be prolonged by transfer of immune serum containing *T. gondii* specific antibodies. This LTβR$^{-/-}$ model clearly shows that both intact T and B cell responses are essential for *T. gondii* containment and survival. This thesis characterizes the role of the LTβR in both innate and adaptive immunity to *T. gondii* in detail and raises important new questions that should be answered in future studies. To date no vaccine to prevent or drug to eradicate human toxoplasmosis is available and a deeper understanding of the molecular mechanisms of LTβR signaling can lead to new insights into the pathophysiology of toxoplasmosis and aid in developing urgently needed new treatment and prevention options such as vaccination strategies for human toxoplasmosis.

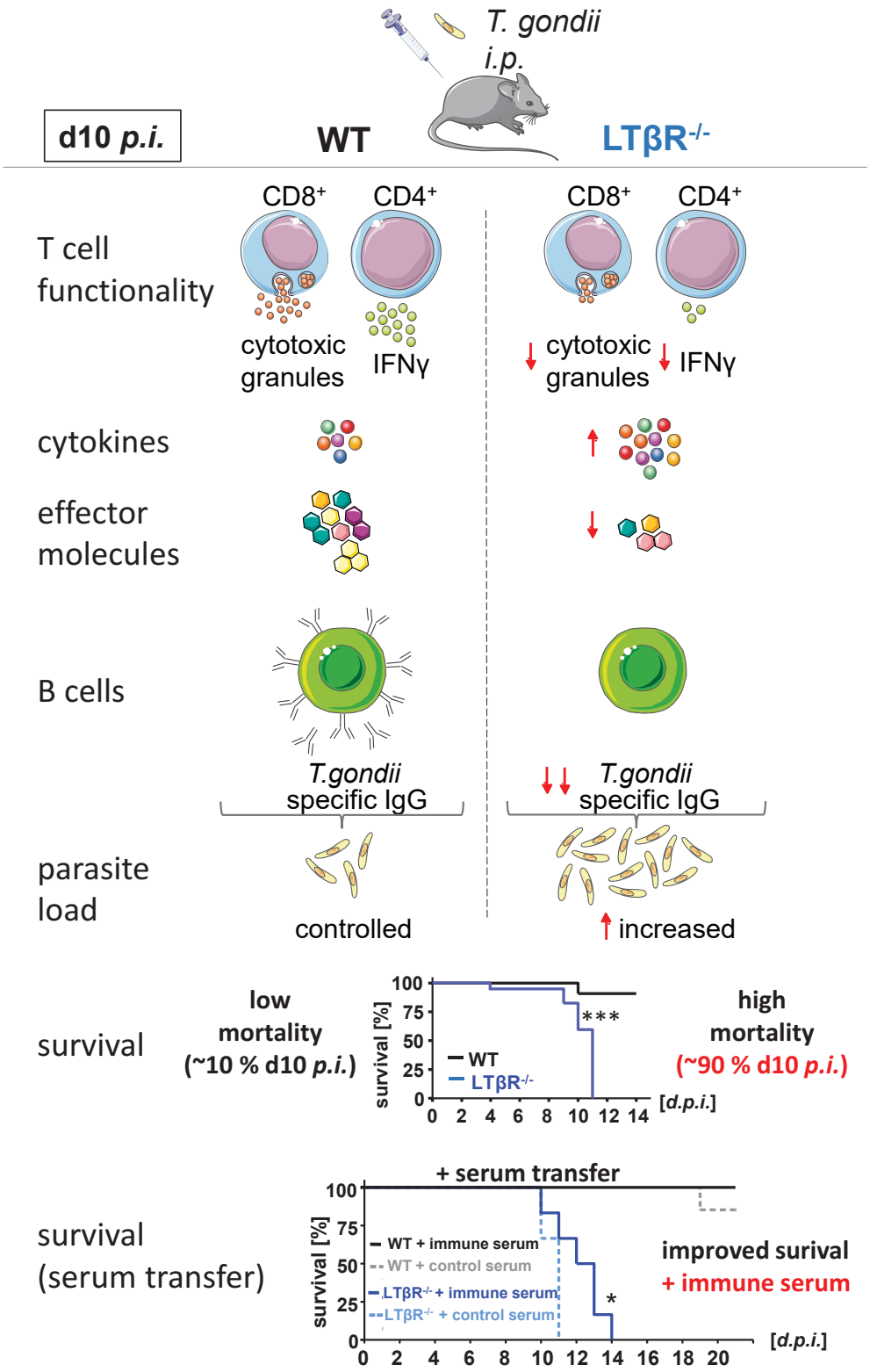

Figure 8: Deficiency of the LTβR affects multiple arms of both innate and adaptive immunity to *T. gondii* infection. Intraperitoneal (*i.p.*) *T. gondii* infection in LTβR$^{-/-}$ mice results in impaired T cell functionality, dysregulated cytokine patterns, decreased IFNγ induced effector molecule responses, and absent parasite specific immunoglobulin (Ig) G antibody response that results in increased parasite numbers causing high mortality of LTβR$^{-/-}$ mice in *T. gondii* infection. However, survival of infected LTβR$^{-/-}$ mice could be prolonged by transfer of immune serum containing *T. gondii* specific antibodies. Red arrows indicate either increased or decreased regulation or expression compared to WT animals on day 10 post infection (*p.i.*). The graphic was designed with Servier Medical Art License (http://creativecommons.org/licenses/by/3.0/). Changes were made to the provided elements.

5. References

Albarbar, B., C. Dunnill and N. T. Georgopoulos (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**(6): 659-671.

Alimzhanov, M. B., D. V. Kuprash, M. H. Kosco-Vilbois, A. Luz, R. L. Turetskaya, A. Tarakhovsky, K. Rajewsky, S. A. Nedospasov and K. Pfeffer (1997). "Abnormal development of secondary lymphoid tissues in lymphotoxin beta-deficient mice." Proc Natl Acad Sci U S A **94**(17): 9302-9307.

Anders, R. A., S. K. Subudhi, J. Wang, K. Pfeffer and Y. X. Fu (2005). "Contribution of the lymphotoxin beta receptor to liver regeneration." J Immunol **175**(2): 1295-1300.

Androlewicz, M. J., J. L. Browning and C. F. Ware (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**(4): 2542-2547.

Ansel, K. M., V. N. Ngo, P. L. Hyman, S. A. Luther, R. Forster, J. D. Sedgwick, J. L. Browning, M. Lipp and J. G. Cyster (2000). "A chemokine-driven positive feedback loop organizes lymphoid follicles." Nature **406**(6793): 309-314.

Banks, T. A., S. Rickert, C. A. Benedict, L. Ma, M. Ko, J. Meier, W. Ha, K. Schneider, S. W. Granger, O. Turovskaya, D. Elewaut, D. Otero, A. R. French, S. C. Henry, J. D. Hamilton, S. Scheu, K. Pfeffer and C. F. Ware (2005). "A lymphotoxin-IFN-beta axis essential for lymphocyte survival revealed during cytomegalovirus infection." J Immunol **174**(11): 7217-7225.

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

Barman, A., B. Deb and S. Chakraborty (2020). "A glance at genome editing with CRISPR-Cas9 technology." Curr Genet **66**(3): 447-462.

Barragan, A. and L. D. Sibley (2003). "Migration of Toxoplasma gondii across biological barriers." Trends Microbiol **11**(9): 426-430.

Behnke, K., U. R. Sorg, H. E. Gabbert and K. Pfeffer (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.

Benedict, C. A., T. A. Banks, L. Senderowicz, M. Ko, W. J. Britt, A. Angulo, P. Ghazal and C. F. Ware (2001). "Lymphotoxins and cytomegalovirus cooperatively induce interferon-beta, establishing host-virus detente." Immunity **15**(4): 617-626.

Beutler, B., Z. Jiang, P. Georgel, K. Crozat, B. Croker, S. Rutschmann, X. Du and K. Hoebe (2006). "Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large." Annu Rev Immunol **24**: 353-389.

Bista, P., W. Zeng, S. Ryan, V. Bailly, J. L. Browning and M. E. Lukashev (2010). "TRAF3 controls activation of the canonical and alternative NFkappaB by the lymphotoxin beta receptor." J Biol Chem **285**(17): 12971-12978.

Bodmer, J. L., P. Schneider and J. Tschopp (2002). "The molecular architecture of the TNF superfamily." Trends Biochem Sci **27**(1): 19-26.

Boehm, U., T. Klamp, M. Groot and J. C. Howard (1997). "Cellular responses to interferon-gamma." Annu Rev Immunol **15**: 749-795.

Bogdan, C. (2015). "Nitric oxide synthase in innate and adaptive immunity: an update." Trends Immunol **36**(3): 161-178.

Bojar, I. and J. Szymanska (2010). "Environmental exposure of pregnant women to infection with Toxoplasma gondii--state of the art." Ann Agric Environ Med **17**(2): 209-214.

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

Browning, J. L., I. D. Sizing, P. Lawton, P. R. Bourdon, P. D. Rennert, G. R. Majeau, C. M. Ambrose, C. Hession, K. Miatkowski, D. A. Griffiths, A. Ngam-ek, W. Meier, C. D. Benjamin and P. S. Hochman (1997). "Characterization of lymphotoxin-alpha beta complexes on the surface of mouse lymphocytes." J Immunol **159**(7): 3288-3298.

Chen, M., H. S. Mun, L. X. Piao, F. Aosai, K. Norose, R. M. Mohamed, U. S. Belal, H. Fang, A. K. Ahmed, H. K. Kang, G. Matsuzaki, D. Kitamura and A. Yano (2003). "Induction of protective immunity by primed B-1 cells in Toxoplasma gondii -infected B cell-deficient mice." Microbiol Immunol **47**(12): 997-1003.

Cheng, H. W., L. Onder, M. Novkovic, C. Soneson, M. Lutge, N. Pikor, E. Scandella, M. D. Robinson, J. I. Miyazaki, A. Tersteegen, U. Sorg, K. Pfeffer, T. Rulicke, T. Hehlgans and B. Ludewig (2019). "Origin and differentiation trajectories of fibroblastic reticular cells in the splenic white pulp." Nat Commun **10**(1): 1739.

Cheung, T. C. and C. F. Ware (2011). "The canonical and unconventional ligands of the herpesvirus entry mediator." Adv Exp Med Biol **691**: 353-362.

Codrici, E., L. Albulescu, I. D. Popescu, S. Mihai, A. M. Enciu, R. Albulescu, C. Tanase and M. E. Hinescu (2018). "Caveolin-1-Knockout Mouse as a Model of Inflammatory Diseases." J Immunol Res **2018**: 2498576.

Coffman, R. L., H. F. Savelkoul and D. A. Lebman (1989). "Cytokine regulation of immunoglobulin isotype switching and expression." Semin Immunol **1**(1): 55-63.

Columba-Cabezas, S., M. Griguoli, B. Rosicarelli, R. Magliozzi, F. Ria, B. Serafini and F. Aloisi (2006). "Suppression of established experimental autoimmune encephalomyelitis and formation of meningeal lymphoid follicles by lymphotoxin beta receptor-Ig fusion protein." J Neuroimmunol **179**(1-2): 76-86.

Courret, N., S. Darche, P. Sonigo, G. Milon, D. Buzoni-Gatel and I. Tardieux (2006). "CD11c- and CD11b-expressing mouse leukocytes transport single Toxoplasma gondii tachyzoites to the brain." Blood **107**(1): 309-316.

Crowe, P. D., T. L. VanArsdale, B. N. Walter, C. F. Ware, C. Hession, B. Ehrenfels, J. L. Browning, W. S. Din, R. G. Goodwin and C. A. Smith (1994). "A lymphotoxin-beta-specific receptor." Science **264**(5159): 707-710.

Darwich, L., G. Coma, R. Pena, R. Bellido, E. J. Blanco, J. A. Este, F. E. Borras, B. Clotet, L. Ruiz, A. Rosell, F. Andreo, R. M. Parkhouse and M. Bofill (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**(3): 386-393.

de Moura, L., L. M. Bahia-Oliveira, M. Y. Wada, J. L. Jones, S. H. Tuboi, E. H. Carmo, W. M. Ramalho, N. J. Camargo, R. Trevisan, R. M. Graca, A. J. da Silva, I. Moura, J. P. Dubey and D. O. Garrett (2006). "Waterborne toxoplasmosis, Brazil, from field to gene." Emerg Infect Dis **12**(2): 326-329.

De Togni, P., J. Goellner, N. H. Ruddle, P. R. Streeter, A. Fick, S. Mariathasan, S. C. Smith, R. Carlson, L. P. Shornick, J. Strauss-Schoenberger and et al. (1994). "Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin." Science **264**(5159): 703-707.

Decker, T., D. J. Lew and J. E. Darnell, Jr. (1991). "Two distinct alpha-interferon-dependent signal transduction pathways may contribute to activation of transcription of the guanylate-binding protein gene." Mol Cell Biol **11**(10): 5147-5153.

Deckert-Schluter, M., H. Bluethmann, A. Rang, H. Hof and D. Schluter (1998). "Crucial role of TNF receptor type 1 (p55), but not of TNF receptor type 2 (p75), in murine toxoplasmosis." J Immunol **160**(7): 3427-3436.

Deckert-Schluter, M., A. Rang, D. Weiner, S. Huang, O. D. Wiestler, H. Hof and D. Schluter (1996). "Interferon-gamma receptor-deficiency renders mice highly susceptible to toxoplasmosis by decreased macrophage activation." Lab Invest **75**(6): 827-841.

Degrandi, D., C. Konermann, C. Beuter-Gunia, A. Kresse, J. Wurthner, S. Kurig, S. Beer and K. Pfeffer (2007). "Extensive characterization of IFN-induced GTPases mGBP1 to mGBP10 involved in host defense." J Immunol **179**(11): 7729-7740.

Degrandi, D., E. Kravets, C. Konermann, C. Beuter-Gunia, V. Klumpers, S. Lahme, E. Wischmann, A. K. Mausberg, S. Beer-Hammer and K. Pfeffer (2013). "Murine guanylate binding protein 2 (mGBP2) controls Toxoplasma gondii replication." Proc Natl Acad Sci U S A **110**(1): 294-299.

Dejardin, E., N. M. Droin, M. Delhase, E. Haas, Y. Cao, C. Makris, Z. W. Li, M. Karin, C. F. Ware and D. R. Green (2002). "The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways." Immunity **17**(4): 525-535.

Denkers, E. Y. (1999). "T lymphocyte-dependent effector mechanisms of immunity to Toxoplasma gondii." Microbes Infect **1**(9): 699-708.

Denkers, E. Y., R. T. Gazzinelli, D. Martin and A. Sher (1993). "Emergence of NK1.1+ cells as effectors of IFN-gamma dependent immunity to Toxoplasma gondii in MHC class I-deficient mice." J Exp Med **178**(5): 1465-1472.

Djurkovic-Djakovic, O., J. Dupouy-Camet, J. Van der Giessen and J. P. Dubey (2019). "Toxoplasmosis: Overview from a One Health perspective." Food Waterborne Parasitol **15**: e00054.

Donley, D. W., A. R. Olson, M. F. Raisbeck, J. H. Fox and J. P. Gigley (2016). "Huntingtons Disease Mice Infected with Toxoplasma gondii Demonstrate Early Kynurenine Pathway Activation, Altered CD8+ T-Cell Responses, and Premature Mortality." PLoS One **11**(9): e0162404.

Dubey, J. P., D. S. Lindsay and C. A. Speer (1998). "Structures of Toxoplasma gondii tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts." Clin Microbiol Rev **11**(2): 267-299.

Dunay, I. R. and A. Diefenbach (2018). "Group 1 innate lymphoid cells in Toxoplasma gondii infection." Parasite Immunol **40**(2).

Ehlers, S., C. Holscher, S. Scheu, C. Tertilt, T. Hehlgans, J. Suwinski, R. Endres and K. Pfeffer (2003). "The lymphotoxin beta receptor is critically involved in controlling infections with the intracellular pathogens Mycobacterium tuberculosis and Listeria monocytogenes." J Immunol **170**(10): 5210-5218.

Endo, T. A., M. Masuhara, M. Yokouchi, R. Suzuki, H. Sakamoto, K. Mitsui, A. Matsumoto, S. Tanimura, M. Ohtsubo, H. Misawa, T. Miyazaki, N. Leonor, T. Taniguchi, T. Fujita, Y. Kanakura, S. Komiya and A. Yoshimura (1997). "A new protein containing an SH2 domain that inhibits JAK kinases." Nature **387**(6636): 921-924.

Endres, R., M. B. Alimzhanov, T. Plitz, A. Futterer, M. H. Kosco-Vilbois, S. A. Nedospasov, K. Rajewsky and K. Pfeffer (1999). "Mature follicular dendritic cell networks depend on expression of lymphotoxin beta receptor by radioresistant stromal cells and of lymphotoxin beta and tumor necrosis factor by B cells." J Exp Med **189**(1): 159-168.

Ettinger, R., S. H. Munson, C. C. Chao, M. Vadeboncoeur, J. Toma and H. O. McDevitt (2001). "A critical role for lymphotoxin-beta receptor in the development of diabetes in nonobese diabetic mice." J Exp Med **193**(11): 1333-1340.

Fava, R. A., E. Notidis, J. Hunt, V. Szanya, N. Ratcliffe, A. Ngam-Ek, A. R. De Fougerolles, A. Sprague and J. L. Browning (2003). "A role for the lymphotoxin/LIGHT axis in the pathogenesis of murine collagen-induced arthritis." J Immunol **171**(1): 115-126.

Feldmann, M. and S. R. Maini (2008). "Role of cytokines in rheumatoid arthritis: an education in pathophysiology and therapeutics." Immunol Rev **223**: 7-19.

Fernandes, M. T., E. Dejardin and N. R. dos Santos (2016). "Context-dependent roles for lymphotoxin-beta receptor signaling in cancer development." Biochim Biophys Acta **1865**(2): 204-219.

Flegr, J., J. Prandota, M. Sovickova and Z. H. Israili (2014). "Toxoplasmosis--a global threat. Correlation of latent toxoplasmosis with specific disease burden in a set of 88 countries." PLoS One **9**(3): e90203.

Force, W. R., B. N. Walter, C. Hession, R. Tizard, C. A. Kozak, J. L. Browning and C. F. Ware (1995). "Mouse lymphotoxin-beta receptor. Molecular genetics, ligand binding, and expression." J Immunol **155**(11): 5280-5288.

Fox, B. A., J. P. Gigley and D. J. Bzik (2004). "Toxoplasma gondii lacks the enzymes required for de novo arginine biosynthesis and arginine starvation triggers cyst formation." Int J Parasitol **34**(3): 323-331.

Fricke, I., D. Mitchell, J. Mittelstadt, N. Lehan, H. Heine, T. Goldmann, A. Bohle and S. Brandau (2006). "Mycobacteria induce IFN-gamma production in human dendritic cells via triggering of TLR2." J Immunol **176**(9): 5173-5182.

Fuglewicz, A. J., P. Piotrowski and A. Stodolak (2017). "Relationship between toxoplasmosis and schizophrenia: A review." Adv Clin Exp Med **26**(6): 1031-1036.

Futterer, A., K. Mink, A. Luz, M. H. Kosco-Vilbois and K. Pfeffer (1998). "The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues." Immunity **9**(1): 59-70.

Gaddi, P. J. and G. S. Yap (2007). "Cytokine regulation of immunopathology in toxoplasmosis." Immunol Cell Biol **85**(2): 155-159.

Gajurel, K., R. Dhakal and J. G. Montoya (2015). "Toxoplasma prophylaxis in haematopoietic cell transplant recipients: a review of the literature and recommendations." Curr Opin Infect Dis **28**(4): 283-292.

Gatumu, M. K., K. Skarstein, A. Papandile, J. L. Browning, R. A. Fava and A. I. Bolstad (2009). "Blockade of lymphotoxin-beta receptor signaling reduces aspects of Sjogren's syndrome in salivary glands of non-obese diabetic mice." Arthritis Res Ther **11**(1): R24.

Gazzinelli, R. T., S. Hieny, T. A. Wynn, S. Wolf and A. Sher (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**(13): 6115-6119.

Gigley, J. P. (2016). "The Diverse Role of NK Cells in Immunity to Toxoplasma gondii Infection." PLoS Pathog **12**(2): e1005396.

Gommerman, J. L. and J. L. Browning (2003). "Lymphotoxin/light, lymphoid microenvironments and autoimmune disease." Nat Rev Immunol **3**(8): 642-655.

Gommerman, J. L., J. L. Browning and C. F. Ware (2014). "The Lymphotoxin Network: orchestrating a type I interferon response to optimize adaptive immunity." Cytokine Growth Factor Rev **25**(2): 139-145.

Gommerman, J. L., K. Giza, S. Perper, I. Sizing, A. Ngam-Ek, C. Nickerson-Nutter and J. L. Browning (2003). "A role for surface lymphotoxin in experimental autoimmune encephalomyelitis independent of LIGHT." J Clin Invest **112**(5): 755-767.

Greenlund, A. C., M. A. Farrar, B. L. Viviano and R. D. Schreiber (1994). "Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91)." EMBO J **13**(7): 1591-1600.

Grell, M. (1995). "Tumor necrosis factor (TNF) receptors in cellular signaling of soluble and membrane-expressed TNF." J Inflamm **47**(1-2): 8-17.

Gresser, I. (1990). "Biologic effects of interferons." J Invest Dermatol **95**(6 Suppl): 66S-71S.

Grigg, M. E., J. Ganatra, J. C. Boothroyd and T. P. Margolis (2001). "Unusual abundance of atypical strains associated with human ocular toxoplasmosis." J Infect Dis **184**(5): 633-639.

Grover, H. S., N. Blanchard, F. Gonzalez, S. Chan, E. A. Robey and N. Shastri (2012). "The Toxoplasma gondii peptide AS15 elicits CD4 T cells that can control parasite burden." Infect Immun **80**(9): 3279-3288.

Halonen, S. K. and L. M. Weiss (2013). "Toxoplasmosis." Handb Clin Neurol **114**: 125-145.
Hartmann, A. (2016). Multiple routes of phosphatidylethanolamine biogenesis ensure membrane integrity of Toxoplasma gondii, Humboldt University Berlin.

Hauer, J., S. Puschner, P. Ramakrishnan, U. Simon, M. Bongers, C. Federle and H. Engelmann (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**(8): 2874-2879.

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

Hinze-Selch, D., W. Daubener, S. Erdag and S. Wilms (2010). "The diagnosis of a personality disorder increases the likelihood for seropositivity to Toxoplasma gondii in psychiatric patients." Folia Parasitol (Praha) **57**(2): 129-135.

Hou, B., A. Benson, L. Kuzmich, A. L. DeFranco and F. Yarovinsky (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**(1): 278-283.

Howard, J. C., J. P. Hunn and T. Steinfeldt (2011). "The IRG protein-based resistance mechanism in mice and its relation to virulence in Toxoplasma gondii." Curr Opin Microbiol **14**(4): 414-421.

Howe, D. K., S. Honore, F. Derouin and L. D. Sibley (1997). "Determination of genotypes of Toxoplasma gondii strains isolated from patients with toxoplasmosis." J Clin Microbiol **35**(6): 1411-1414.

Howe, D. K. and L. D. Sibley (1995). "Toxoplasma gondii comprises three clonal lineages: correlation of parasite genotype with human disease." J Infect Dis **172**(6): 1561-1566.

Hunter, C. A. and L. D. Sibley (2012). "Modulation of innate immunity by Toxoplasma gondii virulence effectors." Nat Rev Microbiol **10**(11): 766-778.

Hunter, C. A., C. S. Subauste, V. H. Van Cleave and J. S. Remington (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**(7): 2818-2824.

Idriss, H. T. and J. H. Naismith (2000). "TNF alpha and the TNF receptor superfamily: structure-function relationship(s)." Microsc Res Tech **50**(3): 184-195.

Ivanova, D. L., S. L. Denton, K. D. Fettel, K. S. Sondgeroth, J. Munoz Gutierrez, B. Bangoura, I. R. Dunay and J. P. Gigley (2019). "Innate Lymphoid Cells in Protection, Pathology, and Adaptive Immunity During Apicomplexan Infection." Front Immunol **10**: 196.

Jin, L., X. Guo, C. Shen, X. Hao, P. Sun, P. Li, T. Xu, C. Hu, O. Rose, H. Zhou, M. Yang, C. F. Qin, J. Guo, H. Peng, M. Zhu, G. Cheng, X. Qi and R. Lai (2018). "Salivary factor LTRIN from Aedes aegypti facilitates the transmission of Zika virus by interfering with the lymphotoxin-beta receptor." Nat Immunol **19**(4): 342-353.

Jones, J. L. and J. P. Dubey (2010). "Waterborne toxoplasmosis--recent developments." Exp Parasitol **124**(1): 10-25.

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

Kaye, P. H., E. Hirst, R. S. Greenaway, Z. Ulanowski, E. Hesse, P. J. Demott, C. Saunders and P. Connolly (2008). "Classifying atmospheric ice crystals by spatial light scattering." Opt Lett **33**(13): 1545-1547.

Khan, I. A., T. Matsuura and L. H. Kasper (1998). "Inducible nitric oxide synthase is not required for long-term vaccine-based immunity against Toxoplasma gondii." J Immunol **161**(6): 2994-3000.

Kim, B. H., A. R. Shenoy, P. Kumar, C. J. Bradfield and J. D. MacMicking (2012). "IFN-inducible GTPases in host cell defense." Cell Host Microbe **12**(4): 432-444.

Kim, E. Y. and K. D. Moudgil (2017). "Immunomodulation of autoimmune arthritis by pro-inflammatory cytokines." Cytokine **98**: 87-96.

Kim, Y. S., S. A. Nedospasov and Z. G. Liu (2005). "TRAF2 plays a key, nonredundant role in LIGHT-lymphotoxin beta receptor signaling." Mol Cell Biol **25**(6): 2130-2137.

Kisseleva, T., S. Bhattacharya, J. Braunstein and C. W. Schindler (2002). "Signaling through the JAK/STAT pathway, recent advances and future challenges." Gene **285**(1-2): 1-24.

Koni, P. A., R. Sacca, P. Lawton, J. L. Browning, N. H. Ruddle and R. A. Flavell (1997). "Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta-deficient mice." Immunity **6**(4): 491-500.

Konstantinovic, N., H. Guegan, T. Stajner, S. Belaz and F. Robert-Gangneux (2019). "Treatment of toxoplasmosis: Current options and future perspectives." Food Waterborne Parasitol **15**: e00036.

Kraaij, M. D., E. J. Vereyken, P. J. Leenen, T. P. van den Bosch, F. Rezaee, M. G. Betjes, C. C. Baan and A. T. Rowshani (2014). "Human monocytes produce interferon-gamma upon stimulation with LPS." Cytokine **67**(1): 7-12.

Kravets, E., D. Degrandi, Q. Ma, T. O. Peulen, V. Klumpers, S. Felekyan, R. Kuhnemuth, S. Weidtkamp-Peters, C. A. Seidel and K. Pfeffer (2016). "Guanylate binding proteins directly attack Toxoplasma gondii via supramolecular complexes." Elife **5**.

Kresse, A., C. Konermann, D. Degrandi, C. Beuter-Gunia, J. Wuerthner, K. Pfeffer and S. Beer (2008). "Analyses of murine GBP homology clusters based on in silico, in vitro and in vivo studies." BMC Genomics **9**: 158.

Krucken, J., J. V. Braun, M. A. Dkhil, A. Grunwald and F. Wunderlich (2005). "Deletion of LTbetaR augments male susceptibility to Plasmodium chabaudi." Parasite Immunol **27**(6): 205-212.

Kursar, M., N. Janner, K. Pfeffer, V. Brinkmann, S. H. Kaufmann and H. W. Mittrucker (2008). "Requirement of secondary lymphoid tissues for the induction of primary and secondary T cell responses against Listeria monocytogenes." Eur J Immunol **38**(1): 127-138.

Kusbeci, O. Y., O. Miman, M. Yaman, O. C. Aktepe and S. Yazar (2011). "Could Toxoplasma gondii have any role in Alzheimer disease?" Alzheimer Dis Assoc Disord **25**(1): 1-3.
Kutsch, S., D. Degrandi and K. Pfeffer (2008). "Immediate lymphotoxin beta receptor-mediated transcriptional response in host defense against L. monocytogenes." Immunobiology **213**(3-4): 353-366.

Lambert, H., N. Hitziger, I. Dellacasa, M. Svensson and A. Barragan (2006). "Induction of dendritic cell migration upon Toxoplasma gondii infection potentiates parasite dissemination." Cell Microbiol **8**(10): 1611-1623.

Lambert, H., P. P. Vutova, W. C. Adams, K. Lore and A. Barragan (2009). "The Toxoplasma gondii-shuttling function of dendritic cells is linked to the parasite genotype." Infect Immun **77**(4): 1679-1688.

Le Poole, I. C., A. I. Riker, M. E. Quevedo, L. S. Stennett, E. Wang, F. M. Marincola, W. M. Kast, J. K. Robinson and B. J. Nickoloff (2002). "Interferon-gamma reduces melanosomal antigen expression and recognition of melanoma cells by cytotoxic T cells." Am J Pathol **160**(2): 521-528.

Lee, S. H., J. Y. Kwon, S. Y. Kim, K. Jung and M. L. Cho (2017). "Interferon-gamma regulates inflammatory cell death by targeting necroptosis in experimental autoimmune arthritis." Sci Rep **7**(1): 10133.

Levy, D. E., D. J. Lew, T. Decker, D. S. Kessler and J. E. Darnell, Jr. (1990). "Synergistic interaction between interferon-alpha and interferon-gamma through induced synthesis of one subunit of the transcription factor ISGF3." EMBO J **9**(4): 1105-1111.

Li, H., Y. X. Fu, Q. Wu, Y. Zhou, D. K. Crossman, P. Yang, J. Li, B. Luo, L. M. Morel, J. H. Kabarowski, H. Yagita, C. F. Ware, H. C. Hsu and J. D. Mountz (2015). "Interferon-induced mechanosensing defects impede apoptotic cell clearance in lupus." J Clin Invest **125**(7): 2877-2890.

Lima, T. S. and M. B. Lodoen (2019). "Mechanisms of Human Innate Immune Evasion by Toxoplasma gondii." Front Cell Infect Microbiol **9**: 103.

Locksley, R. M., N. Killeen and M. J. Lenardo (2001). "The TNF and TNF receptor superfamilies: integrating mammalian biology." Cell **104**(4): 487-501.

Lyons, R. E., R. McLeod and C. W. Roberts (2002). "Toxoplasma gondii tachyzoite-bradyzoite interconversion." Trends Parasitol **18**(5): 198-201.

Mackay, F., J. L. Browning, P. Lawton, S. A. Shah, M. Comiskey, A. K. Bhan, E. Mizoguchi, C. Terhorst and S. J. Simpson (1998). "Both the lymphotoxin and tumor necrosis factor pathways are involved in experimental murine models of colitis." Gastroenterology **115**(6): 1464-1475.

MacKenzie, C. R., K. Heseler, A. Muller and W. Daubener (2007). "Role of indoleamine 2,3-dioxygenase in antimicrobial defence and immuno-regulation: tryptophan depletion versus production of toxic kynurenines." Curr Drug Metab **8**(3): 237-244.

MacMicking, J. D. (2012). "Interferon-inducible effector mechanisms in cell-autonomous immunity." Nat Rev Immunol **12**(5): 367-382.

Mauri, D. N., R. Ebner, R. I. Montgomery, K. D. Kochel, T. C. Cheung, G. L. Yu, S. Ruben, M. Murphy, R. J. Eisenberg, G. H. Cohen, P. G. Spear and C. F. Ware (1998). "LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator." Immunity **8**(1): 21-30.
Meisel, R., S. Brockers, K. Heseler, O. Degistirici, H. Bulle, C. Woite, S. Stuhlsatz, W. Schwippert, M. Jager, R. Sorg, R. Henschler, J. Seissler, D. Dilloo and W. Daubener (2011). "Human but not murine multipotent mesenchymal stromal cells exhibit broad-spectrum antimicrobial effector function mediated by indoleamine 2,3-dioxygenase." Leukemia **25**(4): 648-654.

Mitchell, S., J. Vargas and A. Hoffmann (2016). "Signaling via the NFkappaB system." Wiley Interdiscip Rev Syst Biol Med **8**(3): 227-241.

Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa and V. E. Papaioannou (1992). "RAG-1-deficient mice have no mature B and T lymphocytes." Cell **68**(5): 869-877.

Montoya, J. G. and O. Liesenfeld (2004). "Toxoplasmosis." Lancet **363**(9425): 1965-1976.

Mout, R., M. Ray, Y. W. Lee, F. Scaletti and V. M. Rotello (2017). "In Vivo Delivery of CRISPR/Cas9 for Therapeutic Gene Editing: Progress and Challenges." Bioconjug Chem **28**(4): 880-884.
Murphy, T. L. and K. M. Murphy (2010). "Slow down and survive: Enigmatic immunoregulation by BTLA and HVEM." Annu Rev Immunol **28**: 389-411.

Murray, H. W., A. Szuro-Sudol, D. Wellner, M. J. Oca, A. M. Granger, D. M. Libby, C. D. Rothermel and B. Y. Rubin (1989). "Role of tryptophan degradation in respiratory burst-independent antimicrobial activity of gamma interferon-stimulated human macrophages." Infect Immun **57**(3): 845-849.

Nakano, H., H. Oshima, W. Chung, L. Williams-Abbott, C. F. Ware, H. Yagita and K. Okumura (1996). "TRAF5, an activator of NF-kappaB and putative signal transducer for the lymphotoxin-beta receptor." J Biol Chem **271**(25): 14661-14664.

Nakayama, Y. and J. S. Bromberg (2012). "Lymphotoxin-beta receptor blockade induces inflammation and fibrosis in tolerized cardiac allografts." Am J Transplant **12**(9): 2322-2334.

Nielen, M. M., D. van Schaardenburg, H. W. Reesink, R. J. van de Stadt, I. E. van der Horst-Bruinsma, M. H. de Koning, M. R. Habibuw, J. P. Vandenbroucke and B. A. Dijkmans (2004). "Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors." Arthritis Rheum **50**(2): 380-386.

Nitta, A., H. Suzumura, K. Kano and O. Arisaka (2006). "Congenital toxoplasmosis complicated with central diabetes insipidus in the first week of life." J Pediatr **148**(2): 283.

O'Shea, J. J., M. Gadina and R. D. Schreiber (2002). "Cytokine signaling in 2002: new surprises in the Jak/Stat pathway." Cell **109 Suppl**: S121-131.

Olszewski, M. A., J. Gray and D. J. Vestal (2006). "In silico genomic analysis of the human and murine guanylate-binding protein (GBP) gene clusters." J Interferon Cytokine Res **26**(5): 328-352.

Oygur, N., G. Yilmaz, C. Ozkaynak and A. G. Guven (1998). "Central diabetes insipitus in a patient with congenital toxoplasmosis." Am J Perinatol **15**(3): 191-192.

Pappas, G., N. Roussos and M. E. Falagas (2009). "Toxoplasmosis snapshots: global status of Toxoplasma gondii seroprevalence and implications for pregnancy and congenital toxoplasmosis." Int J Parasitol **39**(12): 1385-1394.

Park, J., J. H. DeLong, J. J. Knox, C. Konradt, E. D. T. Wojno and C. A. Hunter (2019). "Impact of Interleukin-27p28 on T and B Cell Responses during Toxoplasmosis." Infect Immun **87**(12).
Pestka, S., C. D. Krause and M. R. Walter (2004). "Interferons, interferon-like cytokines, and their receptors." Immunol Rev **202**: 8-32.

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**(3): 908-912.

Pfefferkorn, E. R., S. Rebhun and M. Eckel (1986). "Characterization of an indoleamine 2,3-dioxygenase induced by gamma-interferon in cultured human fibroblasts." J Interferon Res **6**(3): 267-279.

Randall, L. M. and C. R. Engwerda (2010). "TNF family members and malaria: old observations, new insights and future directions." Exp Parasitol **126**(3): 326-331.

Rao, A. A., G. R. Sridhar and U. N. Das (2007). "Elevated butyrylcholinesterase and acetylcholinesterase may predict the development of type 2 diabetes mellitus and Alzheimer's disease." Med Hypotheses **69**(6): 1272-1276.

Remouchamps, C., L. Boutaffala, C. Ganeff and E. Dejardin (2011). "Biology and signal transduction pathways of the Lymphotoxin-alphabeta/LTbetaR system." Cytokine Growth Factor Rev **22**(5-6): 301-310.

Saadatnia, G. and M. Golkar (2012). "A review on human toxoplasmosis." Scand J Infect Dis **44**(11): 805-814.

Sanchez, F. M., G. I. Cuadra, S. J. Nielsen, A. Tanner and B. K. Berges (2013). "Production and characterization of humanized Rag2-/-gammac -/- mice." Methods Mol Biol **1031**: 19-26.

Sasai, M., A. Pradipta and M. Yamamoto (2018). "Host immune responses to Toxoplasma gondii." Int Immunol **30**(3): 113-119.

Sasai, M. and M. Yamamoto (2013). "Pathogen recognition receptors: ligands and signaling pathways by Toll-like receptors." Int Rev Immunol **32**(2): 116-133.

Sasai, M. and M. Yamamoto (2019). "Innate, adaptive, and cell-autonomous immunity against Toxoplasma gondii infection." Exp Mol Med **51**(12): 1-10.

Scandella, E., B. Bolinger, E. Lattmann, S. Miller, S. Favre, D. R. Littman, D. Finke, S. A. Luther, T. Junt and B. Ludewig (2008). "Restoration of lymphoid organ integrity through the interaction of lymphoid tissue-inducer cells with stroma of the T cell zone." Nat Immunol **9**(6): 667-675.

Scarzello, A. J., Q. Jiang, T. Back, H. Dang, D. Hodge, C. Hanson, J. Subleski, J. M. Weiss, J. K. Stauffer, J. Chaisaingmongkol, S. Rabibhadana, M. Ruchirawat, J. Ortaldo, X. W. Wang, P. S. Norris, C. F. Ware and R. H. Wiltrout (2016). "LTbetaR signalling preferentially accelerates oncogenic AKT-initiated liver tumours." Gut **65**(10): 1765-1775.

Schaeffer, M., S. J. Han, T. Chtanova, G. G. van Dooren, P. Herzmark, Y. Chen, B. Roysam, B. Striepen and E. A. Robey (2009). "Dynamic imaging of T cell-parasite interactions in the brains of mice chronically infected with Toxoplasma gondii." J Immunol **182**(10): 6379-6393.

Scharton-Kersten, T., H. Nakajima, G. Yap, A. Sher and W. J. Leonard (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**(6): 2565-2569.
Scharton-Kersten, T. M., T. A. Wynn, E. Y. Denkers, S. Bala, E. Grunvald, S. Hieny, R. T. Gazzinelli and A. Sher (1996). "In the absence of endogenous IFN-gamma, mice develop unimpaired IL-12 responses to Toxoplasma gondii while failing to control acute infection." J Immunol **157**(9): 4045-4054.

Scharton-Kersten, T. M., G. Yap, J. Magram and A. Sher (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**(7): 1261-1273.

Schluter, D., W. Daubener, G. Schares, U. Gross, U. Pleyer and C. Luder (2014). "Animals are key to human toxoplasmosis." Int J Med Microbiol **304**(7): 917-929.

Schluter, D., A. Hein, R. Dorries and M. Deckert-Schluter (1995). "Different subsets of T cells in conjunction with natural killer cells, macrophages, and activated microglia participate in the intracerebral immune response to Toxoplasma gondii in athymic nude and immunocompetent rats." Am J Pathol **146**(4): 999-1007.

Schluter, D., L. Y. Kwok, S. Lutjen, S. Soltek, S. Hoffmann, H. Korner and M. Deckert (2003). "Both lymphotoxin-alpha and TNF are crucial for control of Toxoplasma gondii in the central nervous system." J Immunol **170**(12): 6172-6182.

Schneider, K., A. Loewendorf, C. De Trez, J. Fulton, A. Rhode, H. Shumway, S. Ha, G. Patterson, K. Pfeffer, S. A. Nedospasov, C. F. Ware and C. A. Benedict (2008). "Lymphotoxin-mediated crosstalk between B cells and splenic stroma promotes the initial type I interferon response to cytomegalovirus." Cell Host Microbe **3**(2): 67-76.

Schurgers, E., A. Billiau and P. Matthys (2011). "Collagen-induced arthritis as an animal model for rheumatoid arthritis: focus on interferon-gamma." J Interferon Cytokine Res **31**(12): 917-926.

Schwartzman, J. D., S. L. Gonias and E. R. Pfefferkorn (1990). "Murine gamma interferon fails to inhibit Toxoplasma gondii growth in murine fibroblasts." Infect Immun **58**(3): 833-834.

Sedy, J., V. Bekiaris and C. F. Ware (2014). "Tumor necrosis factor superfamily in innate immunity and inflammation." Cold Spring Harb Perspect Biol **7**(4): a016279.

Selleck, E. M., S. J. Fentress, W. L. Beatty, D. Degrandi, K. Pfeffer, H. W. t. Virgin, J. D. Macmicking and L. D. Sibley (2013). "Guanylate-binding protein 1 (Gbp1) contributes to cell-autonomous immunity against Toxoplasma gondii." PLoS Pathog **9**(4): e1003320.

Shao, H., Y. Fu, L. Song, S. Sun, H. J. Kaplan and D. Sun (2003). "Lymphotoxin beta receptor-Ig fusion protein treatment blocks actively induced, but not adoptively transferred, uveitis in Lewis rats." Eur J Immunol **33**(6): 1736-1743.

Shire, J. G. and E. M. Pantelouris (1973). "The adrenals and gonads of congenitally thymusless nude mice." J Endocrinol **58**(1): vii.

Sibley, L. D. and J. C. Boothroyd (1992). "Virulent strains of Toxoplasma gondii comprise a single clonal lineage." Nature **359**(6390): 82-85.

Silveira, E. L. V., M. R. Dominguez and I. S. Soares (2018). "To B or Not to B: Understanding B Cell Responses in the Development of Malaria Infection." Front Immunol **9**: 2961.

So, T. and N. Ishii (2019). "The TNF-TNFR Family of Co-signal Molecules." Adv Exp Med Biol **1189**: 53-84.

Sorg, U. R., K. Behnke, D. Degrandi, M. Reich, V. Keitel, D. Herebian, R. Deenen, M. Beyer, J. L. Schultze, K. Kohrer, H. E. Gabbert, E. Mayatepek, D. Haussinger and K. Pfeffer (2016). "Cooperative role of lymphotoxin beta receptor and tumor necrosis factor receptor p55 in murine liver regeneration." J Hepatol **64**(5): 1108-1117.

Spahn, T. W., C. Maaser, L. Eckmann, J. Heidemann, A. Lugering, R. Newberry, W. Domschke, H. Herbst and T. Kucharzik (2004). "The lymphotoxin-beta receptor is critical for control of murine Citrobacter rodentium-induced colitis." Gastroenterology **127**(5): 1463-1473.

Stanley, A. C., F. de Labastida Rivera, A. Haque, M. Sheel, Y. Zhou, F. H. Amante, P. T. Bunn, L. M. Randall, K. Pfeffer, S. Scheu, M. J. Hickey, B. M. Saunders, C. Ware, G. R. Hill, K. Tamada, P. M. Kaye and C. R. Engwerda (2011). "Critical roles for LIGHT and its receptors in generating T cell-mediated immunity during Leishmania donovani infection." PLoS Pathog **7**(10): e1002279.

Starr, R., T. A. Willson, E. M. Viney, L. J. Murray, J. R. Rayner, B. J. Jenkins, T. J. Gonda, W. S. Alexander, D. Metcalf, N. A. Nicola and D. J. Hilton (1997). "A family of cytokine-inducible inhibitors of signalling." Nature **387**(6636): 917-921.

Steffens, N., C. Beuter-Gunia, E. Kravets, A. Reich, L. Legewie, K. Pfeffer and D. Degrandi (2020). "Essential Role of mGBP7 for Survival of Toxoplasma gondii Infection." mBio **11**(1).

Suzuki, Y. (2019). "The immune system utilizes two distinct effector mechanisms of T cells depending on two different life cycle stages of a single pathogen, Toxoplasma gondii, to control its cerebral infection." Parasitol Int **76**: 102030.

Takaoka, A., Y. Mitani, H. Suemori, M. Sato, T. Yokochi, S. Noguchi, N. Tanaka and T. Taniguchi (2000). "Cross talk between interferon-gamma and -alpha/beta signaling components in caveolar membrane domains." Science **288**(5475): 2357-2360.

Tamada, K., H. Tamura, D. Flies, Y. X. Fu, E. Celis, L. R. Pease, B. R. Blazar and L. Chen (2002). "Blockade of LIGHT/LTbeta and CD40 signaling induces allospecific T cell anergy, preventing graft-versus-host disease." J Clin Invest **109**(4): 549-557.

Taylor, G. A., C. M. Collazo, G. S. Yap, K. Nguyen, T. A. Gregorio, L. S. Taylor, B. Eagleson, L. Secrest, E. A. Southon, S. W. Reid, L. Tessarollo, M. Bray, D. W. McVicar, K. L. Komschlies, H. A. Young, C. A. Biron, A. Sher and G. F. Vande Woude (2000). "Pathogen-specific loss of host resistance in mice lacking the IFN-gamma-inducible gene IGTP." Proc Natl Acad Sci U S A **97**(2): 751-755.

Tedla, Y., T. Shibre, O. Ali, G. Tadele, Y. Woldeamanuel, D. Asrat, A. Aseffa, W. Mihret, M. Abebe, A. Alem, G. Medhin and A. Habte (2011). "Serum antibodies to Toxoplasma gondii and Herpesvidae family viruses in individuals with schizophrenia and bipolar disorder: a case-control study." Ethiop Med J **49**(3): 211-220.

Teixeira, L., A. Marques, C. S. Meireles, A. R. Seabra, D. Rodrigues, P. Madureira, A. M. Faustino, C. Silva, A. Ribeiro, P. Ferreira, J. M. Correia da Costa, N. Canada and M. Vilanova (2005). "Characterization of the B-cell immune response elicited in BALB/c mice challenged with Neospora caninum tachyzoites." Immunology **116**(1): 38-52.

Tenorio, E. P., J. E. Olguin, J. Fernandez, P. Vieyra and R. Saavedra (2010). "Reduction of Foxp3+ cells by depletion with the PC61 mAb induces mortality in resistant BALB/c mice infected with Toxoplasma gondii." J Biomed Biotechnol **2010**: 786078.

Torrey, E. F., J. J. Bartko, Z. R. Lun and R. H. Yolken (2007). "Antibodies to Toxoplasma gondii in patients with schizophrenia: a meta-analysis." Schizophr Bull **33**(3): 729-736.

Tumanov, A. V., D. V. Kuprash and S. A. Nedospasov (2003). "The role of lymphotoxin in development and maintenance of secondary lymphoid tissues." Cytokine Growth Factor Rev **14**(3-4): 275-288.

Ufermann, C. M., A. Domrose, T. Babel, A. Tersteegen, S. C. Cengiz, S. K. Eller, K. Spekker-Bosker, U. R. Sorg, I. Forster and W. Daubener (2019). "Indoleamine 2,3-Dioxygenase Activity During Acute Toxoplasmosis and the Suppressed T Cell Proliferation in Mice." Front Cell Infect Microbiol **9**: 184.

VanArsdale, T. L., S. L. VanArsdale, W. R. Force, B. N. Walter, G. Mosialos, E. Kieff, J. C. Reed and C. F. Ware (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**(6): 2460-2465.

Vestal, D. J., V. Y. Gorbacheva and G. C. Sen (2000). "Different subcellular localizations for the related interferon-induced GTPases, MuGBP-1 and MuGBP-2: implications for different functions?" J Interferon Cytokine Res **20**(11): 991-1000.

Wagage, S., G. Harms Pritchard, L. Dawson, E. L. Buza, G. F. Sonnenberg and C. A. Hunter (2015). "The Group 3 Innate Lymphoid Cell Defect in Aryl Hydrocarbon Receptor Deficient Mice Is Associated with T Cell Hyperactivation during Intestinal Infection." PLoS One **10**(5): e0128335.

Walczak, H. (2011). "TNF and ubiquitin at the crossroads of gene activation, cell death, inflammation, and cancer." Immunol Rev **244**(1): 9-28.

Ward-Kavanagh, L. K., W. W. Lin, J. R. Sedy and C. F. Ware (2016). "The TNF Receptor Superfamily in Co-stimulating and Co-inhibitory Responses." Immunity **44**(5): 1005-1019.

Ware, C. F. (2003). "The TNF superfamily." Cytokine Growth Factor Rev **14**(3-4): 181-184.

Wilson, E. H., T. H. Harris, P. Mrass, B. John, E. D. Tait, G. F. Wu, M. Pepper, E. J. Wherry, F. Dzierzinski, D. Roos, P. G. Haydon, T. M. Laufer, W. Weninger and C. A. Hunter (2009). "Behavior of parasite-specific effector CD8+ T cells in the brain and visualization of a kinesis-associated system of reticular fibers." Immunity **30**(2): 300-311.

Wu, Q., B. Salomon, M. Chen, Y. Wang, L. M. Hoffman, J. A. Bluestone and Y. X. Fu (2001). "Reversal of spontaneous autoimmune insulitis in nonobese diabetic mice by soluble lymphotoxin receptor." J Exp Med **193**(11): 1327-1332.

Wu, Q., Y. Sun, J. Wang, X. Lin, Y. Wang, L. E. Pegg, A. Futterer, K. Pfeffer and Y. X. Fu (2001). "Signal via lymphotoxin-beta R on bone marrow stromal cells is required for an early checkpoint of NK cell development." J Immunol **166**(3): 1684-1689.

Xu, G., D. Liu, I. Okwor, Y. Wang, H. Korner, S. K. Kung, Y. X. Fu and J. E. Uzonna (2007). "LIGHT Is critical for IL-12 production by dendritic cells, optimal CD4+ Th1 cell response, and resistance to Leishmania major." J Immunol **179**(10): 6901-6909.

Yamakawa, R., Y. Yamashita, A. Yano, J. Morita and H. Kato (1996). "Congenital toxoplasmosis complicated by central diabetes insipidus in an infant with Down syndrome." Brain Dev **18**(1): 75-77.

Yamamoto, M., M. Okuyama, J. S. Ma, T. Kimura, N. Kamiyama, H. Saiga, J. Ohshima, M. Sasai, H. Kayama, T. Okamoto, D. C. Huang, D. Soldati-Favre, K. Horie, J. Takeda and K. Takeda (2012). "A cluster of interferon-gamma-inducible p65 GTPases plays a critical role in host defense against Toxoplasma gondii." Immunity **37**(2): 302-313.

Yang, L., J. L. Yang, S. Byrne, J. Pan and G. M. Church (2014). "CRISPR/Cas9-Directed Genome Editing of Cultured Cells." Curr Protoc Mol Biol **107**: 31 31 31-17.

Yap, G. S. and A. Sher (1999). "Effector cells of both nonhemopoietic and hemopoietic origin are required for interferon (IFN)-gamma- and tumor necrosis factor (TNF)-alpha-dependent host resistance to the intracellular pathogen, Toxoplasma gondii." J Exp Med **189**(7): 1083-1092.

Yarovinsky, F. (2014). "Innate immunity to Toxoplasma gondii infection." Nat Rev Immunol **14**(2): 109-121.

Zhai, Y., R. Guo, T. L. Hsu, G. L. Yu, J. Ni, B. S. Kwon, G. W. Jiang, J. Lu, J. Tan, M. Ugustus, K. Carter, L. Rojas, F. Zhu, C. Lincoln, G. Endress, L. Xing, S. Wang, K. O. Oh, R. Gentz, S. Ruben, M. E. Lippman, S. L. Hsieh and D. Yang (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**(6): 1142-1151.

Zhu, S. (2009). "Psychosis may be associated with toxoplasmosis." Med Hypotheses **73**(5): 799-801.

Appendix

List of figures

List of abbreviations

AIDS	acquired immune deficiency syndrome
APC	antigen presenting cell
BAFF	B cell activating factor
BTLA	B- and T lymphocyte attenuator
Cas9	caspase 9
CCL	C-C motif chemokine
CD	cluster of differentiation
cDC	classical dendritic cell
CIA	collagen-induced colitis
CRD	cysteine-rich domain
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DcR3	decoy receptor 3
DC	dendritic cell
d.p.i.	days post infection
EAE	experimental autoimmune encephalomyelitis
e.g.	exempli gratia
FACS	fluorescence-activated cell sorting
fDC	follicular dendritic cell
GAF	interferon gamma activating factor
GAS	interferon gamma activating sequences
GBP	guanylate-binding protein
GSEA	Gene set enrichment analysis
HVEM	herpes virus entry mediator
IDO	indoleamine 2,3-dioxygenase
IFN	interferon

IFNγ	interferon gamma
IFNγR	interferon gamma receptor
Ig	immunoglobulin
IκB	inhibitor of ‘kappa-light polypeptide gene enhancer’ in B cells
IKK	IκB kinase
IKKα	IκB kinase alpha
IKKβ	IκB kinase beta
IKKγ	IκB kinase gamma
IL	interleukin
ILC	innate lymphoid cells
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
IP-10	interferon gamma induced protein 10 kD
IRF-1	interferon regulatory factor 1
IRG	immunity related guanosine triphosphatase
JAK	janus kinase
JNK	c Jun N-terminal kinase
LIGHT	homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes
LT	lymphotoxin
LTi	lymphoid tissue inducer
LTα	lymphotoxin alpha
LTβ	lymphotoxin beta
LTβR	lymphotoxin beta receptor
$LT\beta R^{-/-}$	lymphotoxin beta receptor deficiency
MAPK	mitogen-activated protein kinase
ME	module eigengenes

MEF	murine embryonic fibroblast
mGBP	murine guanylate-binding protein
MHC	major histocompatibility complex
NFκB	nuclear factor ‘kappa-light chain enhancer’ of activated B cells
NIK	NFκB-inducing kinase
NK cells	natural killer cells
NLR	Nod-like receptor
NO	nitric oxide
PAMP	pathogen-associated molecular pattern
PCA	principal component analysis
pDCs	plasmacytoid dendritic cells
PD-L1	programmed death-ligand 1
PLCγ	phospholipase C gamma
p.i.	post infection
PI3K	phosphoinositide 3-kinase
PRR	pattern recognition receptor
pSTAT	phosphorylated signal transducer and activator of transcription
PV	parasitophorous vacuole
ROS	reactive oxygen species
SOCS	suppressor of cytokine signaling
SPF	specified pathogen-free
STAT	signal transducer and activator of transcription
T. gondii	*Toxoplasma gondii*
TLA	toxoplasma lysate antigen
TLR	toll-like receptor
TMD	transmembrane domain
TNF	tumor necrosis factor

TNFα	tumor necrosis factor alpha
TNFR	tumor necrosis factor receptor
TRADD	TNFR-associated death domain
TRAF	TNFR-associated factor
TREX	Transcription and export
Tyr	tyrosine
VLS	vesicle-like structures
WT	wildtype
XAF1	XIAP associated factor 1

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.

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.

_____

Anne Tersteegen

Düsseldorf, 20/07/2020

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