Progranulin Prevents Regulatory NK Cell Cytotoxicity Against Antiviral T Cells

Inaugural dissertation

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presented by

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Contents

Abbreviations

DC: Dendritic cell

NK: Natural killer cells

ILC: Innate lymphoid cells

BM: Bone marrow

IFN-γ: Interferon gamma

LPS: Lipopolysaccharide

IL-4: Interleukin-4

IL-13: Interleukin-13

TNF-a: Tumor necrosis factor alpha

IL-23: Interleukin-23

iNOS: Inducible nitric oxide synthase

ROS: Reactive oxygen species

TGF- β1: Transforming growth factor- β1

ECM: Extracellular matrix

MMPs: Metalloproteinases

CTLs: Cytotoxic T lymphocytes

cDCs: Conventional dendritic cells

pDCs: Plasmacytoid dendritic cells

CDPs: Common dendritic cell precursors

MHC-I: Major histocompatibility complex I

ISGs: Interferon stimulated genes

Th1: T helper cells

cNKs: Conventional NK cells

ILC1: Innate lymphoid cell group 1

ILC2: Innate lymphoid cell group 2

ILC3: Innate lymphoid cell group 3

LTi: Lymphoid tissue inducer cells

MCMV: Murine cytomegalovirus

LP: Lamina propria-resident

MLN: Mesenteric lymph nodes

HSCs: Hematopoietic stem cells

BCR: B cell receptor

TdT: Terminal deoxynucleotidyl transferase

RAG1/2: Recombination activating 1/2

MMPs: Multipotent progenitors

LMPPs: Lymphoid-primed multipotent progenitors

CCL21: Chemokine ligand 21

DN: Double negative stage

DP: Double positive stage

SP: Single positive stage

TCR: T cell receptor

PRRs: Pattern recognition receptors

PAMPs: Pathogen-associated molecular patterns

TLRs: Toll-like receptors

RLR: RIG-I-like receptors

RIG: Retinoic acid inducible gene I

NLR: Nod-like receptors

APC: Antigen-presenting cells

cGAS: Cyclic GMP-AMP synthase

MDA5: Melanoma Differentiation-associated protein 5

dsDNA: Double-stranded DNA

mtDNA: Mitochondrial DNA

cGAMP: Cyclic guanosine monophosphate–adenosine monophosphate

ITIM: Inhibitory receptors contain a tyrpsine-based inhibitory motif

MHC-II: Major histocompatibility complex II

TAP: Transporter associated with antigen processing

CRT: Chaperone calreticulin

PLC: Peptide loading complex

ER: Endoplasmic reticulum

ERAP: ER-anminopeptidase-1

TGN: Trans-golgi network

Li: Invariant chain

CLIP: Class II-associated invariant chain peptides

PKC: Protein kinase C

DAG: Diacylglycerol

PTK: Protein typsine kinase

pMHC: Peptide-MHC complex

IP3: Inositol trisphosphate

PI3K: Phosphoinositide 3 kinase

Tregs: Regulatory T cells

Tfh: Follicular T cells

IPEX: X-linked syndrome

EAE: Experimental autoimmune encephalomyelitis

KLRG1: Killer cell lectin-like receptor G1

LCMV: Lymphocytic choriomeningitis virus

S1P: Subtilisin kexin isozyme 1/site 1 prptease

GP: Glycoproteins

NP: Nucleoprotein

RNP: RNA polymerase to construct ribonucleoprotein

α-DG: α-Dystroglycan

ESCRT: Endosomal sorting complex required for transport

ssRNA: single-stranded RNA

IFN-I: Type I interferon

ppp-ssRNA: 5'-triphosphate single-stranded RNA

CARD: Caspase recruitment domain

IFNAR: Interferon receptor

PD-1: Programmed cell death-1

IRF4: Interferon regulatory factor 4

TCF1: T cell factor 1

Tox: Thymocytes selection-associated high mobility group-boxprotein

FcR: Fc receptor

LGL: Large granular lymphocytes

mNK cells: Mature NK

iNK cells: Immature NK

NCR: Natural cytotoxic receptors

KIRs: Killer cell immunoglobulin-like receptors

NKG2D: Natural killer group 2 D calcium-dependent lectin-like family receptors

MICA/B: MHC class I chain-related gene A/B

GC: Germinal center

TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand

NLRC5: NOD-like receptor caspase recruitment domain containing protein 5

LrNKs: Liver-resident NK cells

PGRN: Progranulin

FTLD: Frontotemporal lobar degeneration

ADAMTS-7: A disintegrin and metalloproteinase with thrombospondin motifs

PR3: Proteinase 3

TNFR: Tumor necrosis factor receptor

EphA2: Ephrin receptor A2

DSS: Dextran sulfate sodium

DTR: Diphtheria toxin receptor

DT: Diphtheria toxin

SPF: Specific pathogen-free

BMDM: Bone marrow derived macrophages

P-TEFb: Positive transcription elongation factor b

AST: Aspartate Aminotransferase

ALT: Alanine Aminotransferase

α-SMA: α-Smooth muscle actin

Declaration and statement of contribution

I, Anfei Huang, declare that the content in this dissertation is original. I have cited appropriate texts and figures wherever necessary. No part of this dissertation is submitted somewhere else for consideration of a degree.

I have contributed in the preparation of following figures which are included in the thesis are as follows,

Fig 1; Fig 2; Fig 4; Fig 5; Fig 6; Fig 7; Fig 8G-I; Fig 10C-D; Fig 11B-D; Fig 12; Fig 13; Fig 14; and Graphical Figures 1-6.

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I was involved in execution of the experiments, analysis, writing and preparation of the manuscript which consists 25% of the published manuscript.

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Date:

Place: Düsseldorf, Germany

Summary

NK cell-mediated regulation of antigen-specific T cells can contribute to and exacerbate chronic viral infection, but the protective mechanisms against NK cell-mediated attack on T cell immunity are poorly understood.

Here, we show that progranulin (PGRN) can reduce NK cell cytotoxicity through reduction of NK cell expansion, granzyme B transcription, and NK cell-mediated lysis of target cells. Following infection with the lymphocytic choriomeningitis virus (LCMV), PGRN levels increased, a phenomenon dependent on the presence of macrophages and type I IFN signaling. Absence of PGRN in mice ($Grn^{-/-}$) resulted in enhanced NK cell activity, increased NK cell-mediated killing of antiviral T cells, reduced antiviral T cell immunity, and increased viral burden, culminating in increased liver immunopathology. However, both naïve or LCMV infected $Grn^{-/-}$ mice showed the comparable NK development and NK cell expression of surface activating or inhibitory receptors to WT mice. In addition, PGRN may not have any effects on virus replication and also on T cell development and activation, suggesting the extrinsic role of PGRN to T cell. Depletion of NK cells restored antiviral immunity and alleviated pathology during infection in $Grn^{-/-}$ mice. In turn, PGRN treatment improved antiviral T cell immunity.

Taken together, we identified PGRN as a critical factor capable of reducing NK cell–mediated attack of antiviral T cells.

List of the figures

1. Introduction

1.1. Immune system

The word "*Immunity*" was firstly described 2500 years ago during the *Peloponnesian War* by *Thucydides*, a well-off Athenian strategist and outstanding ancient Greek historian (1). He described a phenomenon of whom would not be suffered from infectious diseases again if they had been infected before in his work *The Peloponnesian War*. No one could give a scientific explanation of this phenomenon for more than 2000 years until *Louis Pasteur* was born. He firstly showed that pathogens derived from virus or bacterial caused infectious diseases. According to his findings, vaccine science met its opportunity to be improved and widely used. After that, many immune cell types have been identified, such as T/B cells were found in 1960s (2), dendritic cells (DCs) were firstly identified in 1973 (3), and natural killer cells (NK) were identified in 1975 (4). With the joint efforts of many generations of immunologist in the past century, immune systems are divided into two parts, innate immune system and adaptive immune system.

1.1.1. Innate immune system

Epithelial surfaces such as skin, respiratory airway, and gastrointestinal tract build the first line of barriers against invading pathogens. Most of the invading pathogens would be impermeable to these barriers. However, the infectious agents pass over this first line of the defense in case of the skin injuries or tissue damage. The hosts also developed another shield, called innate immunity, to fight against pathogens. The innate immune system is comprised of many types of white blood cells and inflammatory factors (5). The representative cell types of the innate immune cells are macrophages, monocytes, dendritic cells, and innate lymphoid cells (ILCs) (6, 7).

1.1.1.1. Myeloid cells

Macrophages, monocytes, dendritic cells, and granulocytes all belong to the group of phagocytes (8-10). They are identified as the main executor of phagocytosis, which is known to protect the hosts by "eating" or recognizing the harmful external particles, bacterial or even the dying host cells (11-14). Macrophages, which were originally described by *Metchnikoff* in 1893, were found in both lymphoid organs and tissues with morphological and functional diversity. *Van Furth* and colleagues showed that macrophage and monocytes shared the same precursors in bone marrow (BM) and it is terminally differentiated from blood monocytes (15). With the classification of the macrophage phenotype, they were divided into M1 (classically activated macrophages) and M2 (alternatively activated macrophages) categories, which were defined by the response to the interferon-gamma (IFN-γ) and interleukin-4 (IL-4) (16), respectively. M1 activated macrophages display enhanced antibacterial and antitumoral activity by producing high levels of pro-inflammatory cytokines such as IL-6, tumor necrosis factor alpha (TNF-α), and inducible NO synthase (iNOS), which can produce antibacterial chemicals called reactive oxygen species (ROS) and nitrogen radicals (17-19). M1 activation macrophages are associated with tissue injury, whereas M2 activated macrophages displayed wound healing phenotypes after the pathogens had been eliminated (20, 21). M2 activated macrophages in wounds secret transforming growth factor-β1 (TGF-β1) to stimulate epithelial cells and fibroblasts differentiation (22). These epithelial cells and fibroblasts rebuild the first line defense systems by promoting the stability of extracellular matrix (ECM) through enhancing the expression of inhibitors of metalloproteinases (MMPs) (23-25).

Ralph M. Steinman and *Zanvil A. Cohn* identified dendritic cells in peripheral lymphoid organs of mice in 1973 (3). *Michel Nussenzweig* found that DCs are critical accessory cells for the

development of antigen specific cytotoxic T lymphocytes (CTLs) (26). With the contributions to identification and characterization of dendritic cells, *Ralph M. Steinman* was awarded the Nobel Prize in Physiology or Medicine in 2011. With the diversity of function, phenotype and development, distinct DC subpopulations are categorized into two main subsets, conventional DCs (cDCs) and plasmacytoid DCs (pDCs) (27). Both murine cDCs and pDCs are differentiated from common DC precursors (CDPs) in the bone marrows (28-30). cDCs also produce many types of cytokines including IL-12 and 23 in response to invading pathogens (31). cDCs were divided into two main subsets depending on their distinct functions, cDC1 and cDC2 (32, 33). cDC1 exhibits potent processing and cross-presentation of exogenous antigens on major histocompatibility complex I (MHC-I) to activate $CD8^+$ T cells (32, 33), whereas cDC2 is a potent inducer of Th2/17 cell differentiation (34, 35). Viral infection induced type I interferons, which induced many interferon stimulated genes (ISGs) to protect infected cells, are mainly produced by pDCs (36-39). pDCs also have the antigen presenting capacity for both $CD4^+$ and $CD8^+$ T cells (38, 40). With the viral infections or CpG stimulations, pDCs are able to produce IFN-α and IL-12, which induces Th1 differentiation (41, 42).

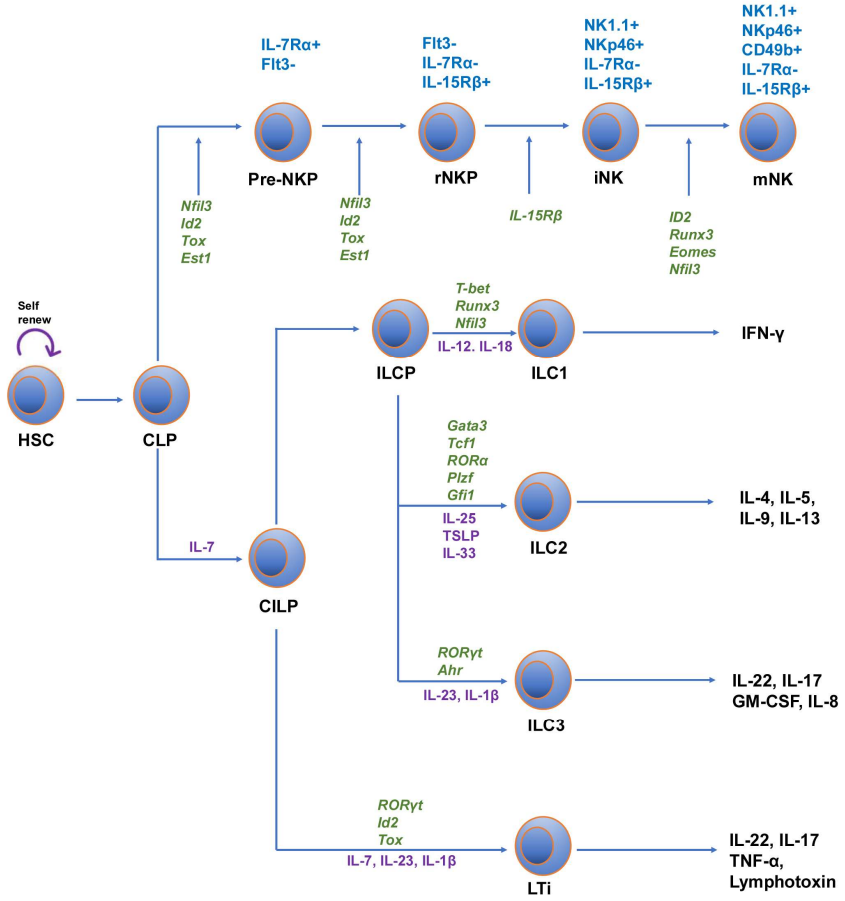
1.1.1.2. Innate lymphoid cells (ILCs)

In 2010, several articles described several new immune cell populations which were not T/B cell-like but NK-cell-like cells (43-46). Since then, scientists classified these new subpopulations as innate lymphoid cells (ILCs) (47, 48). ILCs have been divided into 5 subpopulations depending on their developmental pathways, conventional NK cells (cNKs), lymphoid tissue inducer cells (LTis), group 1 ILCs (ILC1s), group 2 ILCs (ILC2s) and group 3 ILCs (ILC3s) (49-52). The development and cytokines profile of ILCs is summarized in Graphical Figure 1.

ILC1s are potent IFN-γ-producing cells during their activation. IFN-γ is widely known for its role in the immune defense against intracellular pathogens (53). *Christoph Klose* and colleagues showed that ILC1s protect hosts against the infections of intracellular parasite *T. gondii* (54). ILC1s were also described to be the main source of IFN-γ in the early stage of viral infection (55) and therefore played a protective role in murine cytomegalovirus (MCMV) infection (55). In addition, ILC1s in lamina propria-resident (LP), epithelium, and mesenteric lymph nodes (MLN) were the major immune subsets who have the capacity to produce IFN-γ in intestinal pathogen infection (56). Apart from the developmental pathway difference between ILC1 and ILC2, it seems their responses against pathogens are also different. It has been demonstrated that IL-13-producing ILC2s played a protective role in the clearance and prevention of helminth infection (57-59). IL-5/IL-13 co-producing ILC2s control the basal eosinophilopoiesis and tissue eosinophil accumulation, which could be tuned by nutrient intake and central circadian rhythms (60). ILC2s also have a regulatory role in Th2 responses. ILC2-derived IL-13 is a critical factor for the enrichment of $CD40^+$ activated DCs to drain lymph-nodes and subsequently promotes adaptive allergen-induced Th2 immunity (61). ILC3s, like ILC1s, have a protective role for fungus and bacterial infections by being the major source of IL-22 (62-64). Besides their immune defense roles, ILC3s have also been shown to participate in the repair of viral infection- or radiation-induced lymphoid tissue damage (65-67).

The regulatory roles of ILCs in tumor microenvironment have also been implicated. However, the exact role of ILCs in tumor development remains controversial. ILCs have both suppressive and promotive roles in tumor growth, whereas the diversity of their function was dependent on the organs where they were located. Both ILC2s and ILC3s had been demonstrated to promote gastrointestinal cancer development (68-70) and other solid cancers (71-74), whereas they

displayed an anti-tumoral role in melanoma (75, 76) and lung cancer (77, 78). All in all, ILCs play regulatory roles to keep the balance between the immune responses and over-activated innate immunity.



Graphical Figure 1. Figure is depicting the development of NK cells and ILCs. *Adapted from Paola Vacca et al., (79).*

1.1.2. Adaptive immune system

Adaptive immune system is also termed as acquired immune system, which is the direct and potent cleaner of external pathogens. The biggest difference to innate immune system is that the adaptive immune system gives rise to the specific response to a pathogen and subsequently creates

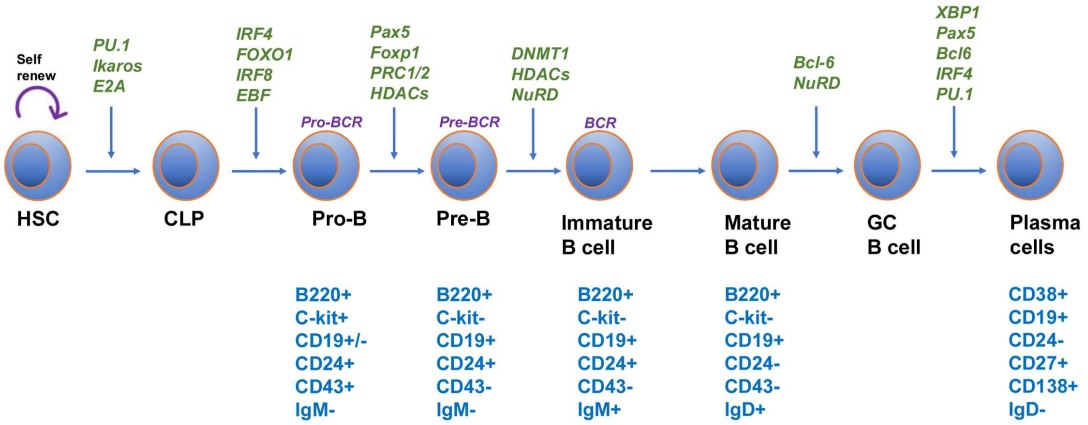
immunological memory. Adaptive immune cells are composed of B lymphocytes and T lymphocytes. The activation of the adaptive immune system is dependent on the innate immune system.

1.1.2.1. B lymphocytes

The pioneers in immunology identified that the circulating antitoxins were the main factors to protect hosts against *diphtheria* and *tetanus* in 1890s (80). After that, immunologists considered the immune cells could release some complementary factors by stimulating with invasions through the receptors on their surface. Until 1939, *Arne Tiselius* and colleagues firstly clarified these antitoxins were γ-globulins, which were subsequently called antibodies, by isolating from serum (81). However, there are still no any evidence to indicate that antibodies were produced by a cellular source until 1948. *Astrid Fagraeus* broke the barrier by indicating that the antibodies were secreted by plasma cells (82). The first antibody-based immunodeficiency disease was reported in 1952, a young boy suffering from multiple infections because of the deficiency of γ-globulins (83). This young boy luckily received treatments by monthly injection of γ-globulins, and had suffered no attack from sepsis (83). This case demonstrated antibodies were the main protective factors to pathogens. In the meantime, immunologists also indicated that the absence of germinal center and plasma cells caused γ-globulin deficiency in humans (84). All these investigations suggested that antibodies were secreted by plasma cells, which represented the terminally differentiated B cells.

Plasma-cell-mediated humoral immunity is an important part against external pathogens. In the beginning of 1970s, *John Owen* and colleagues firstly created a protocol to induce antibody producing cells from fetal liver cells, and they showed that the immunoglobulin expressing cells derived from fetal liver cells were originally immunoglobulin negative (85). In 1974, the other two groups then confirmed that the plasma cells could also derive from small lymphocytes of adult

mouse bone marrow, which express neither surface immunoglobulins nor lymphocyte differentiation antigens (86, 87). Based on these striking findings, hybridoma technology to produce monoclonal antibodies, a revolutionary technique, was firstly created by fusing malignant plasma cells with normal antibody-producing B cells (88). These hybridoma cells can continuously secret antibodies. With the creative efforts by immunologists in the following decades, it is widely accepted that bone marrow resident HSCs guide all stages of B cell development, from earliest pluripotent HSCs to mature B lymphocytes. The molecules expressed on cell surfaces has been widely used for phenotypical characterization of B cell progenitors. Common lymphoid progenitors (CLPs) were firstly identified in the late 1990s, multiple articles showed that a bone marrow subset, that expressing IL-7R, Sca-1, and c-Kit, contains the capacity to differentiate into B cells, T cells and NK cells and subsequently this population was then called Common Lymphoid Progenitors (CLPs) (89-91). Following the CLPs stage, B cell linage was restricted to the expression of B220 and CD19 (89, 91, 92) and was called pre-Pro-B cells and Pro-B cells (92). Pro-B cells become Pre-B cells when the immunoglobulin rearrangement happened, that accompanied by the expression of membrane μ chains, which was considered to be the first step of BCR assembly (93-95). Terminal deoxynucleotidyl transferase (TdT) and recombination activating 1/2 (RAG-1 and RAG-2) were involved in the D-J/V-DJ joining and VDJ rearrangement (96-101). Once the light chain was successfully expressed with μ chain on cell surface, immature B cells were formed (102, 103). The development of B cells is summarized in Graphical Figure 2.



Graphical Figure 2. Figure is depicting the development of B cells. *Adapted from Takashi Nagasawa (104).*

Following the binding with the antigens, immature B cells will face three different fates: (1) cells will be removed if they are nonresponsive to antigens; (2) cells could be deleted if they display over-active responses to self-antigens; (3) cells will migrate to peripheral lymphoid organs from bone marrow to be mature B cells if they could bind to external antigens (105-111).

1.1.2.2. T lymphocytes

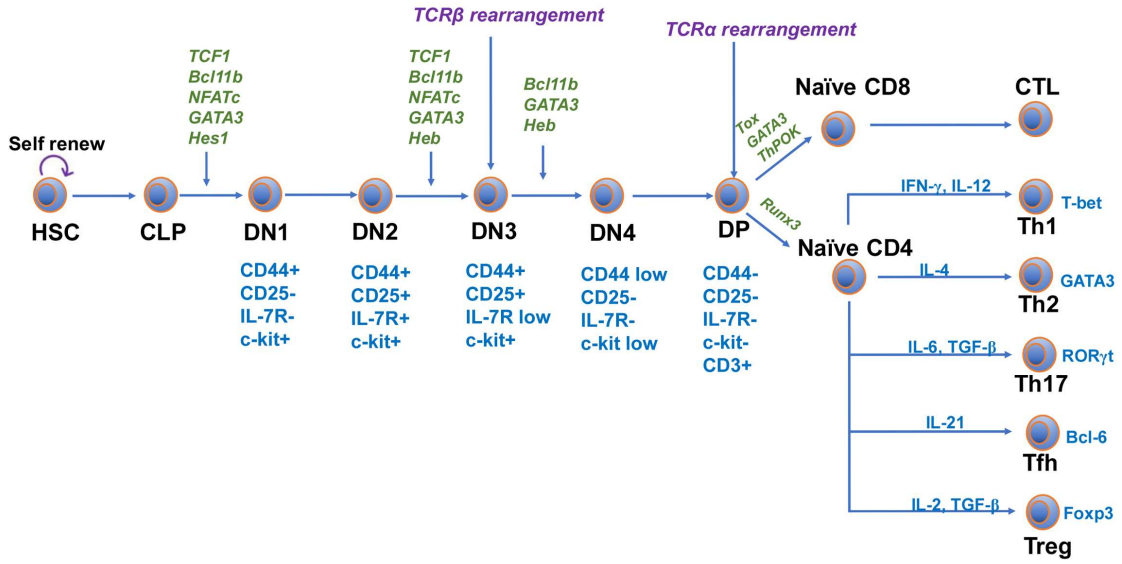
By the 1950s, it was believed that thymus was a redundant organ during evolution (112). In order to protect the breathing from enlarged thymus-induced extrusion of the respiratory tract during surgery, doctors even prescribed irradiation to reduce its size (112, 113). Little was known on the exact role of the thymus until 1961, *Jacques Miller* firstly showed that the thymus had an immunological function and the thymus was essential to life (114, 115). After that, the thymus had been shown to rebuild the immune system (116, 117) and to regulate the antibody formation (118). By transferring the cells from thymus to the recipients, immunologists demonstrated that thymus-derived cells (later known as T cells) can't become plasma cells (later known as B cells) but were

required for helping plasma cells to producing antibodies (2, 119, 120). After that, it was widely accepted that T cells are derived from the thymus.

Reconstitution of irradiated recipients with thymocytes could short-term but not long-term restore the immune system (121), whereas bone marrow cell transfer could restore both long-lived thymocytes and also peripheral T lymphocytes (122, 123). This indicates that thymocytes may be derived from the progenitors in the bone marrow. These progenitors induce several different stages such as HSCs (124-127), multipotent progenitors (MPPs) (128), lymphoid-primed multipotent progenitors (LMPPs) (129, 130), and common lymphoid progenitors (CLPs) (131-133). These progenitors then migrated from the bone marrow into the peripheral circulation system and CLPs finally mobilized into the thymus (134-138). It is known that Notch1 signals control the thymic entry of T cell progenitors (CLPs) (134, 139). Some cell adhesion molecules, like CD44 and CD62L, had also been implicated to regulate the thymic entry of these progenitors. CD44 is widely expressed on HSCs and early T cell progenitors, LMPPs and CLPs (140). The role of CD44 in thymic entry of T cell progenitors was well investigated three decades ago (141). Blocking the progenitors by CD44 antibody prevents the T cell differentiation by intravenous injection but not intra-thymic injection, indicating the regulatory role of CD44 in CLPs thymic entry (142, 143). L-selectin (CD62L) seems to play an indirect role to the thymic migration of T cell progenitors (144). P-selectin glycoprotein ligand-1 (PSGL-1) is highly expressed on multiple progenitors in the bone marrow (145). P-selectin (CD62p), which was highly expressed on thymic endothelial cells, may contract T cell progenitors to the thymus by interaction with PSGL-1 (145, 146). Chemokines also have the capacity to mobilize T cell progenitors to the thymus (147). Chemokines, including stromal-derived factor-1alpha (SDF-1a), Chemokine (CC motif) ligand 21 (CCL21) and CCL25, produced by thymic stromal cells participate the recruitment of progenitors to thymus (148-150).

Before the mature T cells migrate to peripheral organs then undergo 3 stages based on their cell surface CD4/CD8 expression, with starting of $CD4^-$ and $CD8^-$ double negative stage (DN), then becoming $CD4^+CD8^+$ double positive stage (DP) and finally differentiated into $CD4^+$ or $CD8^+$ single positive stage (SP) (151-153). The DN stage is subdivided into 4 substages by cell surface CD25 and CD44 expression (DN1, $CD44^+CD25^-$; DN2, $CD44^+CD25^+$; DN3, $CD44^-CD25^+$; and DN4, $CD44^-CD25^-$) (154). DN1 population is the most potent progenitors to generate T cells, although they just compose 0.01% of total T cells in thymus (154, 155). *TCRβ* and *TCRγδ* gene rearrangement happens at DN2 stage, and this progress will finish at the late DN3 stage (156, 157). IL-7 plays a vital role in TCR rearrangement by recruiting of histone acetylases to the TCRγ locus though IL-7-induced STAT5 signals (158, 159). Therefore, IL-7Rα^{high} DN2 cells were considered to differentiate to γδ T cells, whereas IL-7Rα^{low} cells differentiated to αβ T cells (160). As the same role in BCR rearrangement, *RAG1* displays high levels in DN2 stage cells, which resulted in the surface expression of TCRβ (161). The functional TCRβ or TCRγ chains will be expressed on the DN3 thymocytes (162). Notch1 and CXCR4 signals were reported to induce the correct TCRβ rearrangement (163, 164). Then these cells become $CD25^-$ DN4 thymocytes and finally mature to DP stage thymocytes (165). Several transcription factors had been reported to contribute the whole DN to DP stage transition, including *Runx1* (166), *Runx3* (167, 168), *Bcl11a/b* (169, 170), *Gata-3* (171, 172), *NFAT* (173), and also *E2A* (174, 175). Mature TCR expressing DP thymocytes undergo the positive and negative selection, whereas non-mature or no TCR expressing cells will be sacrificed (176). Positive selection means only the MHC-restricted TCRα/β pair-expressing thymocytes can trigger termination (177, 178). This restriction will induce approximately 90% of DP thymocytes to die (179). The TCR complex will transduce a survival and differentiation signal when they engage a peptide–MHC ligand with low affinity (180, 181). Recognition of a peptide-

MHC I complex selects DP thymocytes to $CD8^+$ SP differentiation, whereas engagements of peptide-MHC II ligand result in $CD4^+$ SP differentiation (182-185). However, there are still lots of thymocytes whose TCR could also bind to self-peptide-MHC ligands. If these cells are terminally differentiated, these SP cells would attack host cells as well. With this issue, the immune system developed a negative selection program, resulting in leading the SP cells apoptosis if they displayed high TCR/peptide-MHC binding affinity (181, 186-188). Functional SP thymocytes will migrate to the circulating system for the terminal differentiation. The development and polarization of T cells are summarized in Graphical Figure 3.



Graphical Figure 3. Figure is depicting the development and polarization of T cells. *Adapted from Divya Shah et al., (189).*

1.1.3. Crosstalk between innate immunity and adaptive immunity

Different types of immune cells from both innate and adaptive immune systems cooperate to maintain the balance between self-tolerance and elimination of invading pathogens or tumorous cells. The innate immune system, including DCs, macrophages and myeloid cells, is programmed

to detect invading pathogens. These innate immune cells are equipped with the so-called pattern recognition receptors (PRRs) to recognize the "nonself" microbial components, which were widely termed as pathogen-associated molecular patterns (PAMPs) (190). The activation of adaptive immunity depends on the signals from the activated innate immunity. Adaptive immune cells always are equipped with a diverse repertoire of BCRs and TCRs, allowing for protection against the constantly evolving pathogens (191). The adequately and suitably crosstalk of innate and adaptive immunity give rise to a balance between the antigen elimination and self-tolerance.

1.1.3.1. The recognition of non-self or stress signals by innate immune system

Several families of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) (192, 193), C-type lectin receptors (194, 195), RIG-I-like receptors (RLRs) (196, 197), and Nod-like receptors (NLRs) (198), are widely expressed on innate immune cells. PRRs recognized not only the nonself signals from the microbes but also the danger signals from the tumor cells or apoptotic cells (199-201). TLRs are the type I transmembrane receptors whose extracellular domains could bind to PAMPs (202). TLRs can be divided into 2 groups based on their cellular localization: cell surface TLRs such as TLR1,2,4,5,6 and endosomal TLRs such as TLR3,7,8,9 (193). The cytosolic viral nucleic acids will be detected by cytosolic sensors like RIG-I/MDA5 in infected cells as well (203-205). Double-stranded DNA (dsDNA) from intracellular bacteria or virus (206, 207), tumor cells (208-210) and also mitochondrial DNA (mtDNA) (211) would be detected by cGAS and then transduces the activation signals to endoplasmic reticulum membrane-resident adaptor STING through the production of cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) (209). Besides pattern recognition, missing-self recognition model is another important strategy for recognizing the nonself features such as the infected or stressed cells, which would be detected by natural killer (NK) cells (212). This strategy is dependent on the

activating and inhibitory receptors on NK cells (213). These receptors may contain a tyrosine-based inhibitory motif (ITIM), which could bind to major histocompatibility complex class I (MHC-I) molecules on healthy and uninfected cells to avoid dysfunction by NK cells (214, 215). Therefore, stressed and viral infected cells would be subjected to NK cell mediated lysis by reducing the cell surface MHC-I expression (216, 217). With these different recognition patterns, innate immunity is activated and ready for priming adaptive immunity.

1.1.3.2. Antigen presentation by antigen presenting cells (APCs)

Antigen presentation is a vital immune process for triggering adaptive immunity. T cell recognition of antigens depends on peptides/MHC complex. $CD8^+$ T cells recognize peptides/MHC-I complex, whereas $CD4^+$ T cells recognize peptides/MHC-II complex (218). Processing of exogenous materials, which was called phagocytosis, is the first step for antigen presentation (219). After the invading pathogens were captured by antigen presenting cells (APCs), the pathogens would be degraded in lysosomes or proteasomes. The maturation of phagosomes is controlled by TLR/MyD88 signals (220, 221). With the degradation by the lysosomes and proteasomes, the external pathogens derived peptides are formed. In general, the peptides generated by proteasomal proteolysis will bind to MHC-I, whereas lysosomal proteolysis produced peptides will bind to MHC-II (222-224).

Peptides binding to MHC-I generally translocate from the phagosomes into the endoplasmic reticulum (ER) through the transporter associated with antigen processing (TAP) (225). The TAP heterodimer, TAP1 and TAP2, associates with several other proteins, such as Tapsin, ERp57 and chaperone calreticulin (CRT) to form the peptide loading complex (PLC) in the ER membranes (226, 227). Cells that lack of TAP1 or TAP2 reduce the peptide/MHC-I complex level (228). Tapasin was used for recruiting MHC-I-β2m dimers and chaperone calreticulin (CRT) to PLC

(229). TAP normally can translocate peptides of more than 40 amino acids length into ERs (230). However, MHC-I can generally capture the peptides 8-11 amino acids in length (231). To yield the peptides of the appropriate length for MHC-I binding, ER aminopeptidase-1 (ERAP1) and ERAP2 are involved to cleave the long peptides to the appropriate length (232). Finally, the mature peptides/MHC-I complex will be transported to the cell membrane.

Different to MHC-I-peptide complex, assembly of peptides-MHC-II complex are taken place in trans-Golgi network (TGN) or late endosomes. MHC-II molecules are constitutively expressed on professional APCs and thymic epithelia. Following synthesis of MHC-II in the ER, invariant chain (Li) protein is expressed and bound to the nascent MHC-II molecules (233, 234). Then the MHC-II-Li complex translocate into the TGN or endosomes, where the invariant chain will be cleaved by cathepsins into class II-associated invariant chain peptides (CLIP) (235, 236). The CLIPs will then be exchanged by the external materials derived peptides from the MHC-II groove with the assistance of MHC-II peptide exchange factor HLA-DM (human) or H2-M (mouse) (237). The MHC-II-peptide complex translocate to the cell membrane for priming $CD4^+$ T cells.

1.1.3.3. T cell activation

In the early 1980s, *Mark M. Davis* and *Tak W. Mak* identified and characterized the antigen receptor of T cells (238, 239). With the efforts of the immunologists in the following decades, a complicated cell surface complex, including CD3γ/δ/ε and the TCRα/β, is shown to be the initiator of T cell activation (240). Until middle 1980s, the activation signals of T cells remained unclear. With the observation that a mutant human T cell line, lacking of TCR, could be activated by the stimulation of phorbol esters and Ca^{2+} ionophores, immunologists speculated that the engagement of TCR/CD3 complex might transduce the same signals to activate T cells (241). This was confirmed by the observations that the intracellular free Ca^{2+} increased following the CD3

antibody or TCR stimulation in T cells (242). It is known that Ca^{2+} increase in T cells is because of the activation of inositol trisphosphate (IP3) (242). Phorbol esters is the activator of protein kinase C (PKC), whereas PKC is regulated by diacylglycerol (DAG) (243). Additionally, both DAG and IP3 could be regulated by phospholipase C (PLC) (244), suggesting the engagement of TCR may transduce the activation signals to PLC. Protein tyrosine kinase (PTK) family members, Lck and Fyn, had also been reported not only to activate PLC activity by TCR or CD4/CD8 T cell coreceptors (245, 246) but also to be the key regulator to T cell activation (247). *Richard D. Klausner's* paper showed that TCR engagement also induced the phosphorylation of ζ chain of the CD3 complex (240). Based on this finding, *Arthur Weiss* showed that a phosphorylated 70 kd protein-tyrosine kinase, pZAP-70, was recruited to the CD3ζ chain in 1992 (248). In summary, with the stimulation of TCR by peptides-MHC complex (pMHC), the Lck kinase is phosphorylated by the signals transduced from the binding of pMHC and CD4 or CD8 coreceptors. Phosphorylated Lck will subsequently phosphorylate CD3ζ and therefore recruit ZAP-70 to the CD3ζ intracellular domain. After the phosphorylation of ZAP-70 occurred, PLC was activated and finally transformed activation signals to IP3 and DAG.

However, if the T cells only get the signals from pMHC, these T cells result in anergy state, which displays functionally inactivated but remains alive for the following hyperactive state to their targets (249). It is suggested that other signals are necessary for functional T cell activation, including costimulatory signals and cytokines which contain also the regulatory roles on T cell activation (250-252). Costimulatory factors may promote or suppress T cell proliferation, cytokines production, and also cell survival (253). The first T cell costimulatory receptor CD28 was identified to enhance TCR stimulation of naïve T cells in 1980s (253). Following binding of CD28 by its ligands CD80/CD86 on antigen presenting cells (APCs), phosphoinositide 3-kinase

(PI3K) will be recruited to the cytoplasmic domain of CD28 and then converts PIP2 to PIP3 (254). Akt protein is phosphorylated by PDK1 (3-phosphoinositide-dependent protein kinase 1), which is recruited by PIP3 (255). Akt can phosphorylate numerous proteins including NF-κB, nuclear factor of activated T cells (NFAT) and GSK-3 (glycogen-synthase kinase 3). NF-κB has positive effects on expression of proteins which could promote T cell survival, such as Bcl-xl (253). However, the costimulatory role of CD28 is not always seen in all T cells. *Arda Shahinian* and colleagues showed in 1993 that CD28-deficient mice displayed reduced T helper cells but normal cytotoxic T cell activity during viral infection (256).

1.1.3.4. $CD4^+$ T cell polarization

Different from the cytotoxic activity of $CD8^+$ T cells, $CD4^+$ T cells mainly play regulatory roles in the immune system. They maintain cytotoxic T cell responses to external antigens but stay in tolerance to self-antigens and contribute to B cell immunity. In the 1980s, two distinctive $CD4^+$ T cell populations were identified based on different cytokines profiles: Th1 cells were characterized with IL2 and IFN-γ production, whereas Th2 cells were characterized with IL-4, IL-5 and IL-13 production (257, 258). Many other $CD4^+$ T cell subpopulations were characterized, such as regulatory T cells (Tregs), Th17 cells and follicular T helper cells (Tfh). Th17, which predominately produce IL-17 and IL-21, were identified in 2003 and well characterized in 2005 (259, 260). In the meantime, Tregs were induced in vitro from the naïve $CD4^+$ T cells by tumor growth factor beta (TGF-β) (261, 262). The recognition of Tfh cells followed the characterization of CXCR5, a chemokine receptor expressed by activated $CD4^+$ T cells but not naïve $CD4^+$ T cells (263). After stimulation with antigens, 50% $CD4^+$ T cells are CXCR5-epxressing cells (264, 265). These $CXCR5^+$ $CD4^+$ T cells from human tonsils displayed a promotive role to facilitate differentiation of naïve B cells into plasma cells (266, 267).

The polarization of T helper cells is determined mainly by the cytokine environment and the transcription factor profile. IL-4-producing Th2 cell differentiation was performed by treating naïve $CD4^+$ T cells with the IL-2 and IL-4 during pMHC stimulation (268). IL-12 remains the central role on the differentiation of IFN-γ-producing Th1 cells (269). Interestingly, IFN-γ itself also worked as an important cytokine for Th1 cell induction with the observation that neutralization of IFN-γ could diminish Th1 cell polarization (270). The combination of TGF-β and IL-6 has been reported to be the most efficient way for Th17 induction in vitro (271, 272). The induction of Tregs from naïve $CD4^+$ T cells also depends on TGF-β in the presence of IL-2 (273). There are many transcription factors that might contribute to the T helper cell development. Gata3, a transcription factor which is critical for $CD4^+$ T cell development, is upregulated when naïve $CD4^+$ T cells differentiate into Th2 cells and downregulated in Th1 differentiation (274, 275). Th2 differentiation is completely diminished in *Gata3*-deficient mice (276). However, overexpressing T-bet in differentiated Th2 cells resulted in enhanced IFN-γ but reduced IL-4 production, suggesting that T-bet might be the major IFN-γ-inducing factor (277). This also has been confirmed in T-bet deficient mice with the observation that *T-bet*-deficient mice displayed diminished IFN-γ response, but increased IL-4 and IL-5 production during infection of *Leishmania major* (278). T-bet promotes IFN-γ expression by remodeling the *IFNG* gene and IL-12 receptor expression (279, 280). Immunodeficiency, polyendocrinopathy, and enteropathy, X-linked syndrome (IPEX) patients have mutations in the *Foxp3* loci, which resulted in undetectable Tregs (281, 282). Foxp3 expression is important for the suppressive activity of Tregs, whereas limitation of Foxp3 expression results in Th2-like cell differentiation (283, 284). RORγT is induced in naïve $CD4^+$ T cells with the stimulation of TCR in association with TGF-β and IL-6, suggesting its promotive role for Th17 development (285). RORγT deficient mice are partly resistant to

experimental autoimmune encephalomyelitis (EAE) (285). Bcl-6 is a transcriptional factor that can efficiently induce the Tfh marker CXCR5 and promote Tfh cell differentiation (286-288). The cytokine and transcription network for T helper cell polarization are summarized in Graphical Figure 3.

1.1.3.5. T cell memory

The T cell response to external antigens can be characterized by three different stages: antigen-specific T cell clonal expansion, effective T cell stage and memory T cell formation. After clonal proliferation of antigen-specific T cells, $CD4^+$ T cells differentiate into Th1 or Tfh cells to coordinate CTL-mediated antiviral immunity and initiate B cell immunity to generate high-affinity neutralizing antibodies. Cytotoxic CTLs that directly destroy virally infected cells by secreting cytotoxic molecules such as granzymes and perforin (289). After the clearance of viral pathogens, ~90% of effector CTLs undergo apoptosis, the other 10% CTLs survive and finally differentiate into memory $CD8^+$ T cells (290). With the viral infection models, the cells that express high IL-7R, CD27 and BCL-2 levels and low killer cell lectin-like receptor G1 (KLRG1) levels have the high potential to differentiated into memory $CD8^+$ T cells (291, 292). However, long-lived $KLRG1^{high}IL\text{-}7R^{high}$ cells can also be detectable in the secondary infections (293-295). Multiple cytokines are also involved in the effector-to-memory transition such as IL-12 and IL-7. Besides the promotive roles for naïve to activated T cell differentiation, IL-12 (p35) also works as a negative regulator in T cell effector-memory transformation (296). At the molecular level, recent studies reveal multiple transcription factors, T-bet, Bcl6, Id3 and Blimp1, have the capacity to regulate the formation of memory T cells. Effector T cells with high levels of T-bet and Blimp1 tend to effector differentiation (297-299), whereas Bcl6 or Id3 expressing effector cells favor terminally differentiated into memory cells (300, 301).

1.2. Lymphocytic Choriomeningitis Virus (LCMV)

In the past decades, mouse models with the infection of Lymphocytic Choriomeningitis Virus (LCMV) have provided many groundbreaking insights into our understanding of infectious diseases. Sickness to LCMV infections is not from the virus itself but from the viral infection induced immune responses. With LCMV infection mouse models, there was a series of great findings that clarified our understanding of defense against pathogens such as the MHC restriction, T cell exhaustion and NK cell mediated antiviral T cell lysis.

1.2.1. LCMV virology

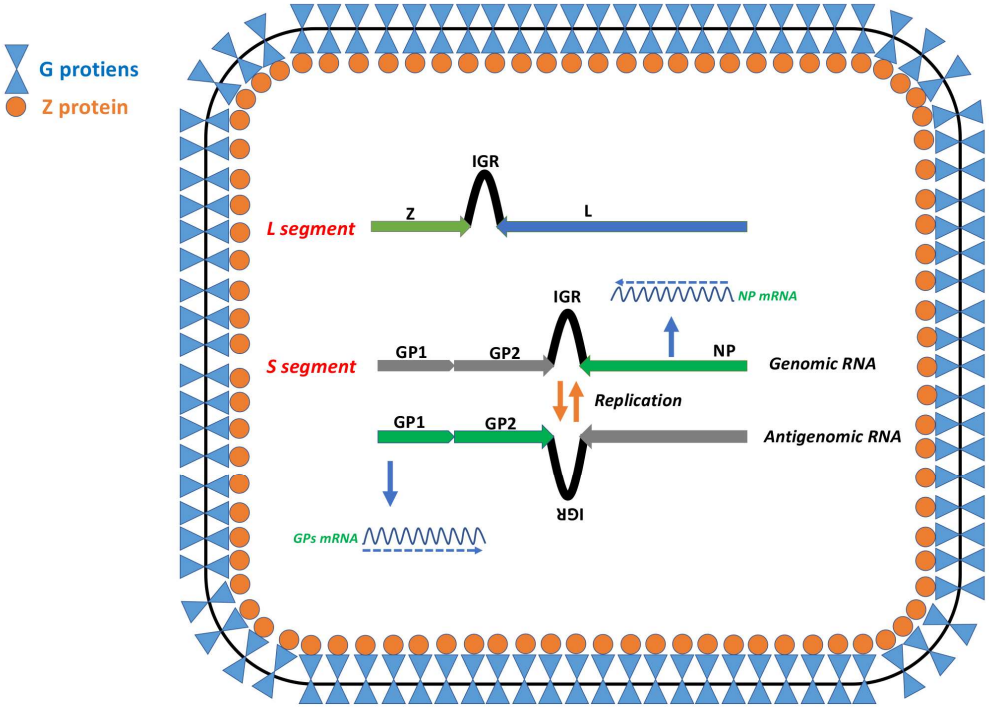
1.2.1.1. LCMV history

LCMV was firstly isolated by *Charles Armstrong* and his colleagues in their study of samples from a *St. Louis encephalitis epidemic* in 1934 (302). In the beginning, they thought the epidemic was caused by LCMV, which was finally uncovered that the epidemic was the infection of Flavivirus St. Louis encephalitis virus (303). Although *Charles Armstrong* was credited with LCMV discovery, the first series of studies of the relationship between LCMV and host immunity were investigated by *Erich Traub* in 1936 (304, 305). It was accepted that virally infected hosts either finally succumbed or survived in a very short period of time. However, newborn mice neither succumbed to LCMV infection nor eliminated the virus (304). It was proposed that the immune system in newborn mice regarded the pathogens from the LCMV as self-antigens. This hypothesis was soon confirmed by the observations that antibodies against LCMV could not be detected in these newborn infected mice (306). This was being argued based on the findings from *Michael Oldstone* that low levels of antiviral antibodies were detectable only in the glomeruli (307). To date, more than 30 LCMV strains have been identified since the first strain *LCMV-Armstrong* was isolated (308). Only six of these strains are widely used in laboratory investigation: Armstrong,

Clone-13, Traub, WE, Aggressive and Docile (309). The Clone-13 strain originated from Armstrong strain with only 5 nucleotides difference (310, 311). The WE strain was also isolated from the samples from the epidemic and known as LCMV-UBC in 1940 (312). The other two strains, Aggressive and Docile were also isolated from the LCMV-UBC strain (313).

1.2.1.2. LCMV Genome

The LCMV virions are enveloped with bilayer lipids and the surface is covered with glycoproteins (Graphical Figure 4). The genome of LCMV virus is composed of two single-stranded RNA fragments, a 3.5 kb S segment and a 7.2 kb L segment (Graphical Figure 4). The shorter S segment encodes a nucleoprotein (NP) and a glycoprotein precursor which can be cleaved into two glycoproteins GP1 and GP2. The RNA polymerase (L protein) and the Z protein (ZP) is encoded by the longer L segment. NP is associated with these two viral RNAs to form the nucleocapsid, which would work together with the viral RNA polymerase to construct the ribonucleoprotein (RNP), which is important for the transcription of other viral proteins and the replication of the viral genome (314). GPs are post-transcriptionally cleaved from the glycoprotein precursor by the protein convertase subtilisin kexin isozyme 1/site 1 protease (S1P) into GP1 and GP2 (315, 316). GP1 coveres the virions and also interacts with the LCMV receptor α-Dystroglycan (DG) (317), whereas GP2 is involved in the fusion of viral envelope with their target cell membrane (318, 319). The function of Z protein remains unclear. It seems that the Z protein inhibits the LCMV mini-genome transcription (320-322). It can also work as a regulatory protein that contribute to virion budding (323, 324). The N-terminal region of ZP contains a conserved RING-finger domain, which is necessary for ZP biological functions (325).



Graphical Figure 4. Figure is depicting the structure and replication of LCMV virus. *Adapted from Sebastien Emonet et al., (326).*

1.2.1.3. LCMV life cycle

The entry of LCMV into cells is initiated by GP1 binding to its cellular receptor α-Dystroglycan (DG), a widely expressed and conserved cell surface molecule which interacts with the extracellular matrix (327). Under the GP1 and α-DG interaction, the virions are taken into a bilayer lipid vesicle and then delivered to the endosomes (328). The late endosomes with an acidic environment promote the exposure of GP2 and therefore initiates the fusion of virions and cell membrane (329, 330). The detailed mechanism of LCMV entry is still not so clear. It is known that the membrane cholesterol is vital for the interaction and entry of LCMV virions and host cell membrane (331). The members of endosomal sorting complex required for transport (ESCRT),

including Hrs, Tsg101 and Vps22, are required for the entry of LCMV virion into the cell membrane (332).

After the fusion of the virion bilayer lipid to endosomal membrane, the virus genome (RNP) is released into the cytoplasm of the host cells. The replication cycle is initiated by the recognition of the highly conserved 3'-terminal of the S segment by the virus L polymerase (333). Since NP is necessary for the genome transcription and replication, the L polymerase transcripts the NP mRNA first. Then the L polymerase continuously moves along the IGR fragment to synthesize the full-length antisense genome S RNA (334, 335). This antisense RNA acts as the template for the amplification of genomic S RNA and the template for the synthesis the mRNA for GPC and the Z protein (325, 336). The replication of the LCMV genome is also summarized in Graphical figure 4.

The assembly and release of LCMV virions occurs at the cell membranes or the early exosome membranes that are enriched with mature viral GPs. The myristoylation modification of proline-rich domains makes the Z protein to be the central role in the budding process during the virus assembly (337, 338). Then the Z protein will be recruited to the cell membrane by Tsg101 (332). Recent studies showed that the NEDD4 family ubiquitin ligases are also necessary for the virus budding process in a Z protein ubiquitination independent way (339, 340). The interaction of GPs, that had been correctly cleaved into GP1 and GP2 by S1P, with Z protein promotes the incorporation of GPs to virus particles (315, 341, 342).

1.2.2. Immune response to LCMV infection

Three strains in the six commonly used strains, including LCMV-Arm, LCMV-WE, LCMV-Aggressive, induced an acute infection in adult mice which is usually eliminated within twenty days, whereas mice infected with the other three strains, LCMV-Traub, LCMV-Docile and

LCMV-Clone 13, resulted in a chronic infection that the virus persists. The immune response to different strains governs the hosts either to eliminate or persistence of LCMV infection.

1.2.2.1. Innate immune responses during LCMV infection

As mentioned in the last section, the viral pathogens are recognized by pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) that are widely expressed on/in antigen presenting cells (APCs) (343, 344). Since both endosomes and the cytosol participate in the LCMV life cycle (331), LCMV can be recognized by endosomal TLRs and RLRs in cytosol (345, 346). During the entry of LCMV into endosomes, TLR7 and TLR8 are responsible to viral single-stranded RNA (ssRNA). However, cell surface TLR2 on glial cells in the central nervous systems (CNS), a type of macrophages, had also been reported to be responsible to LCMV infections (347).

After the entry of LCMV virions into the host cells, the replication cycle is initiated. It will generate 5'-triphosphate single stranded RNA (ppp-ssRNA) and also double stranded RNA (dsRNA), which will be recognized by MDA-5 and RIG-I (346). The interaction of dsRNA/ssRNA with RIG-I/MDA-5 initiates their ATPase/helicase activity resulting in the exposure of their caspase recruitment domain (CARD) to bind to mitochondrial antiviral signalling protein (MAVS) (348, 349). This signal will be transduced to TBK1 and IRF3/7 and finally induce and amplify the IFN-I expression (350).

The critical role of IFN-I in controlling LCMV infection has been described. Absence of IFN-I signaling by IFN receptor (IFNR) deletion results in LCMV virus persistence and also the reduced anti-viral T cell responses (351, 352). Patients with a mutation in the *IFNAR* gene are subjected to either highly susceptible virus replication or succumbed to lethal encephalitis following viral infection (353). In addition, LCMV stays in the central nervous system with

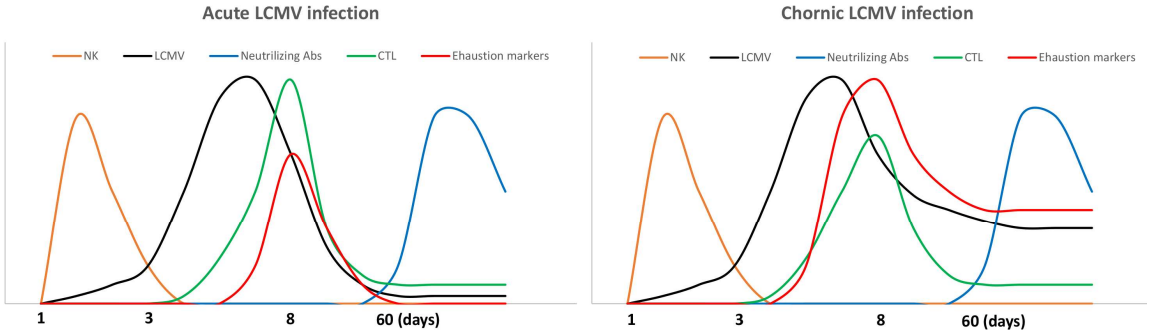
intracranial LCMV-Arm injection in WT mice, whereas the virus spreads to all other organs in *IFNAR-deficient* mice with the same intracranial LCMV infection (354-356). Interestingly, the IFNAR signal has been implicated in facilitating viral persistence with the observation that IFNAR1 blockade initially enhances LCMV-Clone 13 titers at the early timepoint but promotes the clearance of virus at day 30 (357, 358). In addition, following IFNAR1 blockade, mice displayed enhanced anti-viral $CD8^+$ T cells responses and accumulated viral clearance (357, 358).

Secreted IFN-I then promotes the antiviral immunity by inducing the expression of interferon-stimulated genes (ISGs) and therefore amplify the secondary IFN-I secretion. However, induced ISGs may not always have a positive role to viral clearance. For example, the absence of ligoadenylate synthetase-like 1(OASL1), one of ISGs, enhances IFN-I production and therefore promotes the elimination of LCMV-Clone 13 by the enhanced anti-viral T cell responses (359-361). In addition to inducing the expression of ISGs, I-IFNs initiate the activation of other innate immune cells which could play a regulatory role in antiviral innate immune responses such as NK cells (362). *RM. Welsh* firstly showed that NK cells from LCMV infected C3H/St mice contained high levels of cytotoxicity to L929 cells in 1978 (363). Their function during LCMV infection was subsequently identified as activated NK cells play a regulatory role to anti-viral T cells. In the early stage of LMCV infection, activated NK cells attack anti-viral T cells to inhibit T-cell-mediated pathology though natural cytotoxicity triggering receptor 1 (NCR1) (364). IFNs not only activate NK cells but also repress numerous ligands of NCR1 on T cell to protect T cells against NK cell attack (365).

1.2.2.2. LCMV infection induced T cell exhaustion

Although innate immunity initiates the host immune response to virus, the adaptive immunity plays the most straightforward way to eliminate the virus by the cytotoxic responses from antiviral

T cells. Infections of adult mice with LCMV strains that develop acute infection, induces cytotoxic T lymphocyte responses (CTL), whereas prenatally transmitted LCMV or infections of newborn mice result in virus persistence without immune system activation (366, 367). The antiviral T cells can be detected within 7 days, accompanied by the T-cell-mediated pathology at the infection sites (368, 369). Neutralizing antibodies cannot be detected before day 60 after infections, suggesting its suboptimal role in acute infection (370-372). With the infections of LCMV-Docile, LCMV-Clone13 and also LCMV-Traub in adult mice, the virus still can be detected after 100 days. The kinetics of acute and chronic infection induced adaptive immunity are summarized in Graphical Figure 5.



Graphical Figure 5. Figure is depicting the kinetics of immune responses during acute and chronic LCMV infections. *Adapted from Lars Hangartner et al,. (373).*

Although the reason of virus persistence in mice is still not so clear, the viral infection induced T cell exhaustion acts as one of the most important factors. The concept of "exhaustion" of effector T cells was introduced by *Rolf Zinkernagel* in 1993 with the observation of that antiviral $CD8^+$ cytotoxic T cells disappeared within a few days resulted in neither eliminating LCMV virus nor causing lethal immunopathology (374). The exhausted antiviral $CD8^+$ T cells are highly activated but accompanied with enhanced inhibitory molecule expression which resulted in their defective

cytotoxic functions to infected cells (375, 376). Programmed cell death-1 (PD-1) is the most well investigated exhaustion-associated inhibitory receptor, which is transiently highly expressed on the early stage of T cell activation (377, 378). However, PD-1 expression on antiviral $CD8^+$ T cells remains high level during chronic infection (375, 379-381). Therefore, PD-1 blockade restored the exhausted antiviral T cell response with the enhanced proliferation activity, increased cytotoxic potential, and virus elimination (382-384). In addition to expression of exhausted markers on cell surface, the cytokine profile is disrupted as well, such as reduced expression of IFN-γ and TNF-α, which contribute to T cell effector function (385, 386). Exhausted T cells also lose their capacity of producing inflammatory cytokines and their memory differentiation (387).

Besides the genomic difference between chronic and acute LCMV strains, there are many other factors that govern T cell exhaustion during chronic infection, including the duration of high levels of antigen, the status of other immune cells, the cytokines environment, and the transcriptional profile of T cells. It is widely accepted that T cell exhaustion is caused by continuous stimulation with high levels of antigen. *Rafi Ahmed*'s group constructed a bone marrow (BM) chimeric mice, in which WT and MHC-I-deficient hosts were reconstituted with WT BM (388). Interestingly, with the chronic LCMV infection both the numbers and the cytotoxicity of virus-specific $CD8^+$ T cells were increased in this MHC-I-deficient groups, whereas the virus still persisted in control mice. *Dietmar Zehn* also showed that high antigen levels induce an T cell exhaustion in chronic infection by the data in showing that antiviral $CD8^+$ T cells didn't display an exhausted phenotype in mice infected with mutated LCMV-Clone13 virus that lack the gp33 epitope (389). Different doses of LCMV infections also result in different exhaustion status of T cells. Higher viral loads lead to more severe exhaustion and $CD8^+$ T cells exhibits lower cytotoxic activity to virally infected cells (379, 390, 391).

$CD4^+$ T cells play a vital role in initiating the immune responses during both chronic and acute viral infections. The absence of $CD4^+$ T cells result in severe $CD8^+$ T cell exhaustion, suggesting that they are required to sustain the antiviral T cell immunity (392-394). This had also been confirmed by the data showing that $CD4^+$ T cell transfer enhanced the antiviral cytotoxic T cells function during chronic LCMV infection (395). Similar to antiviral $CD8^+$ T cells, $CD4^+$ T cells were also subjected to exhaustion during chronic LCMV infection (396-398). Exhausted $CD4^+$ T cells also display the same characteristics: upregulated inhibitory receptors, lack of functional cytokines expression, and failed to differentiate into memory T cells (396, 397). Distinct from other subsets, $CD4^+$ T cells in chronic infection display a Tfh-like phenotype with the enhanced expression of Bcl6 and CXCR5 (399), suggesting their role in assistance of the B cell response that further eliminate LCMV virus. Regulatory T cells (Tregs) had also been implicated to play a role in the development of chronic LCMV infections by producing immunosuppressive cytokines such as IL-10 and TGF-β (400-402).

The pro-inflammatory cytokine levels not only initiate the innate antiviral immune responses but also contribute to the regulation of T cell exhaustion. As mentioned in the last section, IFN-Is are the major innate antiviral factor for controlling the replication of the virus by inducing ISGs. Blockade of IFN-α altered viral spreading to other organs but failed to eliminate virus, whereas IFN-β blockade promoted the antiviral $CD8^+$ T cell immunity and virus clearance (403), suggesting their distinct roles in LCMV virus persistence. IL-6 is also transiently upregulated at the very early stage of chronic infection and increased again at around 25 days after chronic infection (404). IL-6 had been shown in supporting Tfh cell responses against infections and also in supporting $CD4^+$ T cell IL-21 expression (405). $CD4^+$ T cells are the main source of IL-21 and IL-21 signaling deficiency impeded the LCMV virus control by inducing severe T cell exhaustion

in mice (406-408). In contrast, recombinant IL-21 treatment improved the antiviral T cell responses (407). Tumor necrosis factor (TNF) is another proinflammatory cytokine associated to T cell exhaustion during chronic infection. Blockade of TNF, as well as TNF receptor deficiency, rescued antiviral T cell numbers and their cytotoxic functions (409).

Many T cell intrinsic molecules are also involved in the progress of T cell exhaustion. Several new transcription factors have been reported to be associated with the T cell exhaustion in chronic LCMV infections. Interferon Regulatory Factor 4 (IRF4) is a transcription factor, which was shown to be highly expressed in exhausted antiviral CD8 T cells (375). IRF4 promoted expression of inhibitory receptors by directly binding to their promoters with other two transcription factors BATF and NFAT, which has also been shown in controlling T cell exhaustion during chronic viral infection (410, 411). The T-box transcription factors family members T-bet and Eomes not only play a regulatory role in T cell and innate immune cell (ILC) development (412, 413), but also a crucial role in T cell exhaustion during chronic infection (293, 299, 414, 415). T-bet worked as a repressor of PD-1 expression by directly binding to its promoter and therefore suppressed the T cells differentiation into an exhausted phenotype (416). Another transcription factor Blimp1 may indirectly suppress PD-1 expression through inhibiting the transcription factor NFAT1, which could bind to the PD-1 promoter as well (417). T cell factor 1 (TCF1) impedes T cell exhaustion by augmenting Eomes expression and regulating Bcl2 levels to maintain exhausted T cell survival (418-420). Similar to the role of other transcription factors in exhausted cells, the thymocyte selection-associated high mobility group-box protein (Tox) also promotes the T cell function in chronic infections (421-424). All in all, the combination of T cell extrinsic and intrinsic factors depict the progress of T cell exhaustion during LCMV chronic infection.

1.3. The role of NK cells in LCMV infection

Natural killer (NK) cell plays a crucial role in both innate and adaptive immunity by mediating spontaneous and MHC-I non-restricted cytotoxicity to pathogen-infected cells, malignantly transformed cells, and over-activated immune cells through their cytolytic machinery including the cell surface receptors and secretory molecules such as granzymes and perforin (425-429). In this chapter, NK cells are described in the following sections: their discovery, development, receptors and their regulatory role in viral infections.

1.3.1. NK cell biology

1.3.1.1. NK cell history

With the findings from *Ralf Zinkernagel* and *Peter Doherty* in 1974, it is well accepted that cytotoxic T lymphocytes (CTLs) recognize antigens on virally infected cells or tumor cells in a MHC restricted way (430). Interestingly, studies of human lymphocytes mediated cytotoxicity in 1970s revealed that allogeneic tumor cells could also be lysed in a non-MHC-restricted way, suggesting the existence of some immune cell subsets containing a natural cytotoxic activity (431-435). The same finding in mouse models was also reported at the meantime by observations that spontaneous cytotoxicity of naïve mouse splenocytes attacking leukemia cells was caused by a small undefined lymphocyte population which were termed as natural killer (NK) cells (4). NK cells were defined by lacking of T/B lymphocyte markers but highly expressing Fc receptor (FcR) since the technical limitation in 1970s (436-441). The concept of NK cells was not widely accepted by all immunologists since the lack of unique markers until the first evidence that NK cell cytotoxicity was highly associated with a unique lymphocyte population called large granular lymphocytes (LGLs) (442, 443). After this milestone NK cells were then purified from the PBMC culture by the presence of IL-2 (444). With the efforts from the immunologists in the following

decades, more and more NK cell specific markers have been identified on both human and mouse NK cells. Human NK cells are identified by $CD56^{+}CD3^{-}TCR^{-}$ and $CD56^{+}$ NK cells can be further divided into $CD56^{bright}$ and $CD56^{dim}$ subpopulations (445, 446). $CD56^{dim}$ NK cells produce less cytokines but contain higher cytotoxic activity to target cells than $CD56^{bright}$ NK cells (446). Mouse NK cells are characterized as $CD49b^{+}NK1.1^{+}CD3e^{-}$ and can be delineated into four subpopulations based on the CD27 and CD11b expression during NK cell activation (447).

1.3.1.2. NK cell homeostasis

Although NK cells are mainly developed in the bone marrow, recent studies revealed that NK cells can also differentiate from peripheral lymphoid organs including lymph nodes (LNs) (448), spleen (449), liver (450), thymus (451), and tonsils (452). Since we are using a mouse model in our study, we will mainly discuss about the development of murine NK cells. The self-renewing hematopoietic stem cells (HSCs) firstly give rise to the common lymphoid progenitors (CLPs), which can also differentiate into T and B lymphocytes. CLPs then give rise to CD122-expressing NK progenitors (NKP) (453). Interleukin (IL)-7 and IL-15 that are produced by mesenchymal stromal cells (MSCs) and fibroblastic reticular cells promote an important role in programming CLPs into NKPs (454). In addition, IL-21-producing MSCs facilitate the expansion of NKPs (455). The maturation of iNK cells was associated by reticular cell-producing CXCL12 through its binding to NKP cell surface CXCR4 (456). Mature NK cells (mNK) then migrate to peripheral lymphoid organs in a non-activated state. Functional NK cell maturation can be divided into three subpopulations: $CD27^{+}CD11b^{-}$, $CD27^{+}CD11b^{+}$, $CD27^{-}CD11b^{+}$ (447). CD11b single positive stage ($CD27^{-}CD11b^{+}$) is considered to be the terminally matured NK cells (457, 458), whereas the $CD27^{+}CD11b^{+}$ double positive stage exhibits the most effective cytotoxic activity to their targets (447).

In the past decades, several NK-lineage specific transcription factors in NK cell development have been identified, including leucine zipper protein Nfil3 (also known as E4BP4) (459, 460), E protein antagonist Id2 (461, 462), high mobility group box protein Tcf1 (463, 464), thymocyte selection associated high mobility group box (Tox) (465, 466), and Ets family protein Ets1 (467). Unlike other transcription factors, the absence of E4BP4 resulted in only severe NK cell lineage reduction but not T lymphocytes and B lymphocytes (459, 460), suggesting its specific role to NK cell homeostasis. The development of NK cells is also summarized in Graphical Figure 1.

1.3.1.3. NK cell receptors

NK cells are important innate effector cells for protecting the hosts against infected cells, malignantly transformed cells, the stressed host cells, and also cells from other hosts through granules mediated exocytosis or death receptor pathways induced apoptosis (468). Early studies showed that the activation of NK cells was induced by the target cells that lack of expression of MHC class I (469), suggesting NK cells were activated by non-self signals (it was called the missing-self hypothesis). Cells that express the host MHC class I molecules are resistant to NK cells, whereas cells expressing without or non-self MHC class I molecules are subjected to the NK cell mediated lysis (426). The recognition of "non-self" cells is mediated by many types of inhibitory or activating receptors on the NK cell surface, including natural cytotoxic receptors (NCR), natural killer group 2 calcium-dependent lectin-like family receptors (NKG2), and killer cell immunoglobulin-like receptors (KIRs) (470, 471).

Natural cytotoxic receptor (NCR) represents a group of activation receptors including NKp30, NKp44, and NKp46 (NCR1). NKp46 is specifically expressed on NK cells, whereas NKp30 and NKp40 had also been revealed to be expressed on T cells (472). NCRs are immunoglobulin-like type I transmembrane glycoproteins that contain a positively charged amino acid in their

intracellular domains, which can assist the signal transduction to their signaling partners (473, 474). NKp46 and NKp30 are commonly thought to associate with FcRγ chain, whereas NKp44 transduces signals to DAP12 (475). Normally, an immunoreceptor-based activation motif (ITAM) locates in the cytoplasmic tail of the signaling partner (476).

NKG2 family receptors work as the ligands that bind to nonclassical MHC class I molecules on the surface of potential target cells (477). NKG2D is one of the best studied NKG2 family receptors and is expressed on all NK cells. Mouse NKG2D can transduce signals to two signal partners DAP10 and DAP12, whereas human NKG2D associates DAP10 (478-480). Many NKG2D ligands had been identified, including MHC class I chain-related genes (MICA, MICB) and UL-16 binding proteins (ULBP) in human cells (481-484), Rae-1, H60 and MILTI-1 in mouse cells (485). These NKG2D ligands remain a basal level in healthy tissues, but are highly expressed on the virally infected or malignantly transformed cells (486, 487). The expression of these ligands on stressed or infected cells initiates the NK cell cytotoxicity. On the other hand, NK cells usually express lower NKG2D in cancer patients, which is mediated by immuno-suppressive cytokines such as IL-10 and TGF-beta which is produced by suppressive immune cells or tumor cells in the tumor microenvironment (488). Other NKG2 family members share the same models for NK activation or suppression, such as NKG2A contains a ITIM domain which leads to the inhibition of NK cell activity, whereas NKG2C/E/H associates DAP12 and results in the antiviral or antitumoral activity of NK cells (477).

1.3.2. The role of NK cells in LCMV infection

LCMV infections induce strong cytokine-driven NK cell activation that limits the response of antiviral T cells. The observation that adaptive immune cell could be the target of NK cells has long been known for decades (489-491). Immature thymocytes are resistant to NK cells since NK

cells remain in an inactivated state in normal physiological conditions. After LCMV infection NK cells will be activated and will attack both the infected cells and activated immune cells. Fortunately, viral infections induce type I IFNs, which increases the MHC class I molecule expression on thymocytes and protects antiviral T cells against NK cell cytotoxicity (492, 493). In addition, naïve mature T cells remain resistant to NK cell. LCMV induced IFN-I suppress many types of ligands to NK cell activating receptors (494, 495). Antiviral T cell that lack IFN receptor cannot escape NK cell mediated lysis *in vivo* during LCMV infection due to increased NCR1 ligand expression (364, 365, 496). Depletion of NK cell during LCMV infections results in enhanced antiviral T cell responses. Thereby virus elimination and immune pathology during LCMV infection is imposed (497-499). In addition to antiviral T cells, NK cells also contain profound immune effects on germinal center (GC) B cells with the observations that NK cell deficiency results the enhanced Tfh cell responses, that promote GC B cell responses and therefore induce more antiviral antibody secretion (500). A recent study reveals that most of T cell subsets are sensitive to NK cell mediated lysis (501).

Many other molecules have been shown to regulate NK-cell-mediated T cell lysis during viral infections. Most of them may directly regulate NK cell activity. The absence of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) leads to improved antiviral $CD8^+$ T cell responses, resulting in faster LCMV clearance and reduced liver pathology (502). Similarly, FcRγ stabilizes the expression of NKp46 (NCR1) expression on NK cells to regulate NK cell mediated T cell lysis during LCMV infection (503). CD47 acts as a cell-intrinsic regulator of NK cell function in response to a LCMV infection (504). Blockade of CD47 with an antibody leads to increased antiviral $CD8^+$ T cell responses (505), suggesting its regulatory role of the crosstalk between NK cells and T cells. On the other hand, there are also studies showing the molecules

which could regulate MHC-I expression on T cells and therefore protect T cells against NK cell cytotoxicity. NOD-like receptor caspase recruitment domain containing protein 5 (NLRC5) deficiency suppresses T cell MHC-I expression, resulting in enhanced NK-cell-mediated T cell lysis (506).

Which NK cell subpopulations are responsible to T cell lysis has not been investigated yet. The study from *Jing Zhou* and colleagues revealed that liver-resident NK cells (LrNKs), marked as CD49a, but not CD49b conventional NK cells (cNKs) retained the main activity to attack antiviral T cells in mice during LCMV infections (507).

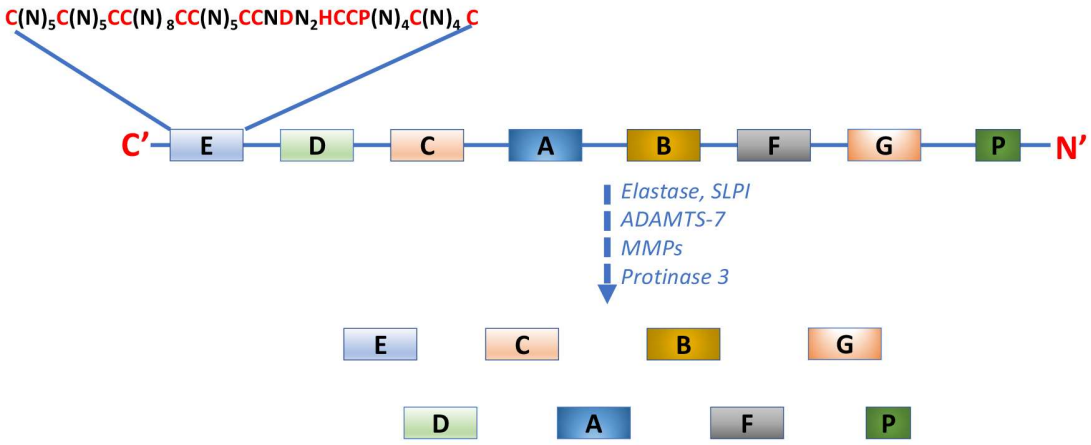
1.4. Progranulin

Progranulin (PGRN) is also named as pro-epithelin, granulin-epthelin precursor, PC-cell-derived growth factor, and acrogranin (508). PGRN has been shown to be associated with diverse pathological conditions and physiological processes. With the studies of PGRN in the past decades, numerous therapeutic strategies targeting PGRN have been generated.

1.4.1. PGRN biology

Studies of PGRN in the past decades highlight that PGRN may have different roles in various kinds of diseases, including neurological diseases, inflammatory disease, cancer, wound healing, and rare lysosomal storage diseases (509).

PGRN was firstly described in the 1990s with its role in tumor growth, nerves cell growth, and wound healing (510-512). The links between mutations in *GRN* (encodes PGRN) and frontotemporal lobar degeneration (FTLD) was shown in 2006 (513, 514). PGRN is a cysteine-rich secreted protein that contains 7.5 repeats of granulin peptide motifs (Graphical Figure 6) (515-517). Secreted PGRN could undergo cleavage in the extracellular space by proteases including elastase, a disintegrin and metalloproteinase with thrombospondin motifs 7 (ADAMTS-7), proteinase 3 (PR3), and matrix metalloproteinases (MMPs), whereas the PGRN cleavage can be suppressed by secretory leukocytes protease inhibitor (SLPI) (518). Interestingly, PGRN cleavage products with different cutting sites may have opposing effects on cell growth (519). It can also be cleavaged in the late lysosomes by cathepsin family proteases (520, 521).



Graphical Figure 6. Figure is depicting the structure of Progranulin protein. *Adapted from Sharon Demorrow (522).*

PGRN is widely expressed in both hematopoietic cells and non-hematopoietic cells (523). The MAPK and Akt signalling pathway were activated in mouse embryo fibroblasts (MEFs) by the stimulation with recombinant PGRN, suggesting there might be some cell surface PGRN receptors (524). Several surface molecules have been implicated to be the functional receptors to PGRN, including Sortilin, TNF receptor 1, and EphA2. The absence of Sortilin reduced PGRN binding to microglial cells, resulting in increased soluble PGRN in the brain and serum with sortilin assisted lysosomal endocytosis of PGRN as a mechanism (525). PGRN has also been reported to directly competitively bind to TNF receptors to disturb the TNFα-TNFR interaction in rheumatoid arthritis (526). However, *Xi Chen* and colleagues showed that PGRN cannot bind to TNF receptors and also was not the direct regulator of TNF signaling in both immune and neuronal cells (527). To provide perspective to this controversy, *Etemadi et al.* tested the ability of recombinant PGRN from different commercial sources to inhibit TNF signaling. Unfortunately, no inhibitory activities of TNF signaling could be observed by PGRN in this case (528). However,

the inhibition of TNF signaling has been confirmed by Atsttrin, an engineered protein composed of three PGRN fragments, by several studies (529-531), suggesting PGRN may not directly interact with TNF receptors. Furthermore, Ephrin receptor A2 (EphA2) shows high affinity to recombinant PGRN, resulting in activation of MAPK and Akt signaling pathways (532).

1.4.2. The role of PGRN in disease

1.4.2.1. PGRN in cancers

Various cancers express higher levels of PGRN than non-tumor tissues, including cancers in liver (533), breast (534), ovary (535, 536), prostate (537), kidney (538), and also in brain (539), implying it could be used for the diagnosis and prognosis of cancer. PGRN may promote tumor growth in many ways such as promoting cancer cell proliferation (511, 540), invasion (533, 541), and resistance to cytotoxic cells (542). PGRN rendered liver cancer cells resistant to NK cell cytotoxicity by modulating the expression of MICA, which is the ligand of NK cell activating receptor NKG2D (542). A PGRN monoclonal antibody was also generated to suppress hepatocellular carcinoma development (543), indicating PGRN is a functional target for hepatocellular carcinoma.

PGRN not only directly promotes cancer cell growth but also modulates the angiogenesis during wound healing by inducing human microvascular endothelial cell proliferation and the tube-like structure formation (544). In addition, tumor PGRN levels are correlated to the VEGF levels, an endothelial growth factor that induce angiogenesis (545). The stimulation of recombinant PGRN promotes cancer cell VEGF expression, whereas knockdown or deletion of PGRN results in downregulation of angiogenic factors in colorectal carcinoma cells (545).

Numerous PGRN targeting strategies for tumor therapy had been developed in the past decades by inhibiting PGRN expression with siRNA, anti-sense cDNA, or specific neutralizing

antibodies. The tumorigenicity was significantly disturbed by transducing the tumor cells with the anti-sense PGRN cDNA *in vivo* (546-548). Similarly, knockdown of PGRN by siRNA leads to slow tumor growth (549). Furthermore, treatment with PGRN specific neutralizing antibodies results in inhibition of hepatocellular carcinoma development and breast cancer cell growth (543, 550, 551). Macrophage-secreted PGRN may play an important role in pancreatic cancer liver metastasis by inducing liver fibrosis (552). All these findings suggest that PGRN could be used as a potential tumor therapy target.

1.4.2.2. PGRN in autoimmunity

It has long been reported that PGRN plays a role in autoimmunity, including rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, psoriasis, diabetes mellitus, systemic erythematosus, and multiple sclerosis (553). The absence of PGRN in mice results in a higher incidence and more severe clinical features of arthritis, whereas the disease progression can be rescued by the treatment of recombinant PGRN (526). PGRN deficiency in TNF-transgenic mice leads to accelerated inflammatory arthritis (526). Furthermore, rheumatoid arthritis serum shows a higher PGRN protein level, as compared to healthy controls (554-556). The ratio of PGRN to TNF has been reported to correlate with the rheumatoid arthritis stage (556). The suppressive role of PGRN is thought to promote the activity of Tregs (526, 557). A similar phenotype was also observed in osteoarthritis, that PGRN-deficient mice exhibited a severe osteoarthritis and both recombinant PGRN and Atsttrin (Granulins) protected mice against osteoarthritis (558, 559).

The elevated levels of PGRN have been shown in serum of human inflammatory bowel disease and mouse colitis models induced by dextran sulfate sodium (DSS) (560). PGRN may have a protective role in inflammatory bowel disease, evidenced by the observations that PGRN deficient mice are highly susceptible to DSS-induced inflammatory bowel disease and treatments

with recombinant PGRN result in reduced disease severity by the mechanism of modulating the balance between Tregs and Th17 cells (560).

1.4.2.3. PGRN in infectious diseases

PGRN is also highly expressed in neutrophils, that are critical for host defense in bacterial infections (561). Neutrophil-derived elastase converts PGRN to Granulins, which may recruit neutrophils to the infection sites by inducing the expression of IL-8 from the epithelial cells (561). PGRN deficient mice failed to efficiently eliminate bacteria by inhibiting the recruitment of monocytes (562). Gastric epithelial cells can secrete PGRN during direct contact between bacteria and cells (549, 563), suggesting the existence of sensors that can bind to PGRN. *Boyoun Park* and colleagues revealed that granulins were an essential secreted cofactor that assisted the recruitment of nucleotides to TLR9 and therefore modulating the anti-bacterial innate immunity (564).

PGRN also binds to the cysteine-rich domain of HIV transactivator Tat protein (565), implying that PGRN contains a regulatory role to HIV replication and transcription. Cyclin T1 is an important constituent of transcription elongation factor P, which directly interacts with Tat protein (566). PGRN interacts with Cyclin T1 to suppress HIV transcription and replication in vitro (567, 568). Taken together, PGRN plays an important role in innate immunity.

2. Aim and hypothesis of this thesis

Millions of human die every year since the viral infection diseases. It is urgent to clearly understand the mechanisms of viral infection induced death. LCMV is a widely used virus in mouse models for investigating the relationship between host immune responses and viral infections.

Progranulin is widely known by its role in central nervous system disease, *GRN* mutations resulted into a variety of clinical features, causing mostly behavioral frontotemporal dementia and progressive non-fluent aphasia. Progranulin had also been identified as the upregulated molecule during LCMV infection in mouse in our preliminary investigations, suggesting that it might play functional roles during viral infections.

By LCMV infection models, we notified that virus in $Grn^{-/-}$ mice cannot be cleared as fast as that in WT control mice, suggesting the protective role of progranulin during viral infection. However, the mechanism of this phenotype remains unclear. There are three possibilities of why progranulin deficiency results in slower viral clearance: 1. Progranulin protein directly suppresses virus propagation; 2. Progranulin promotes innate immunity to enhance host innate antiviral immunity such as promoting more type I interferons, etc; 3. Progranulin directly promotes adaptive antiviral immunity such as cytotoxic T cell activity. With our experimental evidence we notified that progranulin has no effects on virus propagation, slight impact on cytokines production and no promotive role on cytotoxic T cell activity.

NK cell is also known as an important regulator to adaptive T cell response during LCMV infection through direct T cell lysis. In our study, progranulin showed potent suppressive role on NK cell expansion and activation. Therefore, the hypothesis of this study is that viral infections

induced progranulin directly suppresses NK cell activation and subsequently promotes enhanced antiviral cytotoxic T cell immunity and finally accelerates virus clearance.

3. Materials and methods

3.1. Animals

Grn$^{-/-}$ (B6[Cg]-*Grn*$^{tm1.1\text{Aidi/J}}$) mice, which were previously described (The Jackson Laboratories, stock no. 01375) (562), were used and compared with C57BL/6J mice. *Rag1*$^{-/-}$, *Ifnar1*$^{-/-}$, CD169-DTR, CD11c-DTR, and CD45.1^{+}P14 mice were maintained under specific pathogen-free (SPF) conditions. For chimera experiments, WT mice were irradiated with 10.5 Gy. One day later, mice were reconstituted with BM cells from indicated donors. Six- to 8-week-old age-matched and sex-matched mice were used for all experiments. All mice used in this study were maintained in a standard barrier facility at Heinrich-Heine-University Düsseldorf.

3.2. Virus

LCMV strain WE was originally obtained from F. Lahmann-Grube (Heinrich Pette Institute, Hamburg, Germany). LCMV-WE was propagated in L929 cells as previously described (569). Virus titers were determined using a plaque-forming assay as previously described (570).

3.3. Reagents

Mouse-specific antibody to CD3ε (145-2C11, 11-0031-85), CD3e (17A2, 47-0032-82), NKp46 (29A1.4, 11-3351-82), CD11b (M1/70, 47-0112-82), IL-7R (A7R34, 11-1271-85), 2B4 (ebio244F4, 25-2441-82), CD5 (53-7.3, 47-0051-82), CD8 (53-6.7, 47-0081-82), CD19 (eBio1D3, 47-0193-82), Ly6G (RB6-8C5, 47-5931-82), TCR-β (H57-597, 47-5961-82), FcεR1 (MAR-1, 47-5898-82), Emoes (Dan11mag, 61-4875-82), CD4 (GK1.5, 48-0041-82), and GATA3 (TWAJ, 50-9966-42) from eBioscience were used. Mouse-specific antibody to NK1.1 (PK136, 25-5941-82), CD49b (DX5, 17-5971-81), granzyme B (NGZB, 12-8898-82), perforin (eBioOMAK-D, 11-9392-82), IFN-γ (XMG1.2, 17-7311-82), Tim3 (RMT3-23, 12-5870-82), CD16/32 (93, 11-0161-82),

NKG2D (CX5, 25-5882-82), Ly49F/C/I/H (14B11, 12-5991-81), and Ter119 (TER119, 47-5921-82) were from Invitrogen. Mouse-specific antibody to CD27 (LG.3A10, 563365), CD44 (IM7, 563736), CD69 (H1.2F3, 561238), KLRG1 (2F1, 740553), CD62L (MEL-14, 563117), PD-1 (J43, 562523), CXCR5 (2G8, 563981), Lag3 (C9B7W, 563179), NKG2A/C/E (20d5, 740153), and RORγT (Q31-378, 562607) were from BD Biosciences. Human-specific antibody to CD56 (NCAM, 318328) was from BioLegend; IFN-γ (4S.B3, 11-7319-82), CD3e (OKT3, 45-0037-42), CD14 (61D3, 45-0149-42), CD19 (SJ25C1, 45-0198-42), and CD16 (eBioCB16, 47-0168-42) were from eBioscience; and CD107a (H4A3, 561348) was from BD Biosciences. Inhibitor (SNS-032) against cyclin T1/CDK9 was purchased from Selleck Chemicals (S1145). Mouse PGRN ELISA kit (EMGRN) and granzyme B (catalog 88-8022-22) were purchased from Invitrogen. Foxp3 mouse Treg Staining Buffer Set (eBioscience, 00-5523-00) was used. NK cell isolation kits (catalog 130-052-501) were from Miltenyi Biotec. Cell Proliferation Dye eFluor 450 (Invitrogen, 65-0842-85) was used for NK cell, Rma, and RMA/S cell labeling. Apoptotic cells or dead cells were stained with 7-AAD (Invitrogen, 00-6993-50) and Annexin V (BD Biosciences, 550474) in Annexin V staining buffer (BD Pharmingen, 51-66121E). Complete protease inhibitor cocktail (MilliporeSigma, 329-98-6) was used to lysate NK cells. RNeasy Mini Kits (250) (QIAGEN, 74106) were used for RNA extraction. Elastase was used for PGRN digestion to granulin (MilliporeSigma, E8140). CDK9 (Cell Signaling Technology, 2316S), cyclin T1 (Abcam, ab184703), phosphor-Ser2-RNA polymerase II (pSer2-R II, Abcam, ab193468) and α-SMA (Abcam, ab32575) antibodies were used. Anti–Rat IgG (Jackson ImmunoResearch, 112-116-072) were used for LCMV-NP staining; Cy3-conjugated goat anti–rabbit IgG (Jackson ImmunoResearch, 111-165-144) was used for cyclin T1 and phosphor-RNA polymerase II (pSer2-R II) flow cytometry staining. Horseradish peroxidase–conjugated (HRP-conjugated) goat anti–

rat IgG (Jackson ImmunoResearch, 112-035-003) was used for plaque assasys. IRDye 800CW goat anti–rabbit IgG secondary antibody (LI-COR, 926-32211) was used for Western blotting (see complete unedited blots in the supplemental material). HRP-conjugated rabbit anti–β-actin antibody was used for Western blotting (Cell Signaling Technology, 5125S). PageRuler Prestained Protein Ladder (Thermo Fisher Scientific, 26617) was used. Recombinant mouse IL-2 (Miltenyi Biotec, 130-12-333); mouse IL-12 (BioLegend, 577002), mouse IL-15 (Peprotech, 210-15), and mouse IL-18 (BioLegend, 767002) were used. PMA (MilliporeSigma, P8139) and ionomycin calcium salt (MilliporeSigma, I0634) were used for T cell activation.

3.4. Enzyme-linked immunosorbent assay (ELISA)

The serum from infected mice were collected for examining the PGRN levels at day 1 after infection (LCMV-WE). Granzyme B levels in the supernatant of NK cell cultures were measured by granzyme B ELISA kit. All experiments were performed according to the manufacturers' instructions.

3.5. Recombinant PGRN purification

Stable PGRN-expressing HEK-293T cells were used. The supernatants from these cells had been collected and HiTrap TALON (GE Healthcare) crude columns, and AktaPrime were used for PGRN purification according to the manufacturers' instructions.

3.6. Cell isolation and culture

Single-cell suspended splenocytes were enriched following the manufacturer's instructions with the DX5 MACS kit (Miltenyi Biotec). Isolated NK cells were expanded by 1,000 IU/ml IL-2 with or without indicated concentration of PGRN in RPMI-1640 (containing 10% FBS, 2 mM L-glutamin, and 100 U/ml penicillin-streptomycin) for 4 days in a humidified cell culture incubator at 37°C with 5% CO_2. Vero, N2a neuroblastoma, and L929 cells were cultured in Eagle's

Minimum Essential Medium (Hyclone) supplemented with 10% FBS and 100 U/ml penicillin-streptomycin (Thermo Fisher Scientific) in a humidified cell culture incubator at 37°C with 5% CO_2.

3.7. Flow cytometry

Tetramer and intracellular cytokine staining were performed as described previously (569). For intracellular staining, Foxp3 mouse Treg staining buffer sets were used according to the manufacturer's protocol. ILC and NK cell stainings were performed as previously described (571). Experiments were performed using a FACS Fortessa (BD Bioscience) and analyzed with FlowJo software (Treestar).

3.8. NK cell cytotoxicity assays

Freshly insolated NK cells were expanded by IL-2 for 4 days. Susceptible RMA/S and RMA cells were labeled by 10 μM Cell Proliferation Dye eFluor 450 ($eF450^+$) and exposed to NK cells at indicated effectors/targets ratios. 18 hours later, 7-AAD was added, and the percentage of $7\text{-}AAD^+$ cells among $eF450^+$ target cells was determined by flow cytometry. Control and $Grn^{-/-}$ mice were infected with LCMV-WE strain (2×10^6 pfu) for 24 hours. Splenocytes were collected and mixed with RMA/S cells at indicated effector/target ratio for 18 hours. 7-AAD was added, and the percentage of $7\text{-}AAD^+$ cells among $eF450^+$ target cells was determined by flow cytometry.

3.9. Cell depletion

NK cells were depleted with i.v. injections of anti-NK1.1 (clone PK136) as previously described (572). For depletion of macrophages, mice were treated with Clodronate liposomes (200 μl), and control mice were treated with empty control liposomes (200 μl) as previously described (573). For $CD11c^+$ and $CD169^+$ cell depletion, CD11c-DTR and CD169-DTR mice were injected with 0.01 mg/kg body weight diphtheria toxin (DT) for 24 hours.

3.10.Immunofluorescence

Histological analysis of snap-frozen tissue was performed as previously described by using anti-α-SMA and self-made anti-LCMV-NP monoclonal antibody (clone VL4) (571). NK cell cyclin T1 was stained using the Foxp3 staining kit, and these cells were cytospun onto slides. Cells were visualized with the Zeiss fluorescence microscope after staining with 4,6-diamidino-2-phenylindole.

3.11.Quantitative PCR

RNA purification was performed according to the manufacturer's instructions (QIAGEN). Gene expression analysis of *Cdk9*, *Ccnt 1*, *Gzmb*, *Grn*, and Prf1 were performed using kits from Applied Biosystems. All primers are listed in *Table 1*. For analysis, the expression levels of all genes were normalized to GAPDH or Actin. Then, gene expression values were calculated based on the ΔΔCt method relative to controls.

Table 1. Primers used in this study.

Genes	Forward ((5'-3')	Reverse ((5'-3')
CDK9	GTACGACTCGGTGGAATGCC	GATGGGGAACCCCTCCTTCT
Ccnt1	ATGCCTGATCGTACCGAGAAG	GTCGTTGGCGTAAATGAGCTG
Gzmb	CCACTCTCGACCCTACATGG	GGCCCCCAAAGTGACATTTATT
Prf1	AGCACAAGTTCGTGCCAGG	GCGTCTCTCATTAGGGAGTTTTT
Grn	GTGTTGTGAGGATCACATTC	CTATGACCTTCTTCATCCAG
Gapdh	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC
Actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

3.12.Immunoblotting

NK cells were lysed with lysis buffer (20 mM Tris-HCL, pH 7.5; 0.5% Nonidet P-40; 10 mM NaCl; and 3 mM EDTA) containing complete protease inhibitor cocktail for 30 minutes on ice.

Cell lysates were separated by SDS-PAGE and analyzed with immunoblotting. Immunoblots were probed with primary antibody: cyclin T1, PGRN, CDK9, and β-actin, followed by secondary antibody or enhanced chemiluminescence detection of fluorescence secondary antibody, and detected by LI-COR imager (Odyssey Fc, LI-COR Biosciences).

3.13.Bone marrow derived macrophages (BMDM) culture

BM cells were collected and cultured in DMEM supplemented with 10% FBS and 20% L929 cell culture supernatant. At day 7, differentiated BMDMs were treated with LCMV-WE (MOI=1.0) for 48 hours. The levels of PGRN in these supernatants were measured by ELISA.

3.14.Statistics

Data are expressed as mean ± s.e.m. For analysis of statistical significance between 2 groups, a Student's *t test* (2-tailed) was used. For the analysis of human NK cell data, paired Student's *t test* (2-tailed) was used. For the analysis of multiple time point experiments, 2-way ANOVA was used; a P value less than 0.05 was considered significant. Statistical analysis was performed using GraphPad Prism.

3.15.Study approval

Cryopreserved PBMCs from 15 healthy donors were used in this study. Informed consent was obtained from each donor, and the study protocol was approved by the local ethics committee of the University Hospital Dusseldorf (Ethical number is NR: 2018-131-KFogU). Animal experiments were performed under the authorization of Landesamt für Natur, Umwelt, und Verbraucherschutz Nordrhein-Westfalen (LANUV) in accordance with German law for animal protection.

4. Results

4.1. Recombinant PGRN suppresses NK cell expansion

Although PGRN has long been investigated in central nervous system diseases, its role on immune cells remains unclear. In order to test whether PGRN had direct effects on NK cells, we exposed murine NK cells to different doses of recombinant human PGRN. Interestingly, the presence of PGRN reduced the IL-2-mediated expansion of NK cells in a dose-dependent manner (Figure 1A and B).

To understand how PGRN suppresses NK cells expansion, the apoptosis of these NK cells was measured. Flow cytometry analysis revealed that PGRN treated NK cells displayed comparable level of cell apoptosis to untreated groups (Figure 1C), suggesting PGRN may not suppress NK cell expansion by inducing cell apoptosis. However, the cell cycles were disturbed by PGRN treatment by the observation that PGRN treatment increased the presence of NK cell in G0/G1 stage and reduced proportion of NK cells in S and G2/M phases (Figure 1D). The expression of many NK cell surface markers, including NKG2D, CD69, CD11b, CD27, CD44, and NKG2A/C/E, were also measured during NK cell expression with or without PGRN treatment. Flow cytometry analysis indicated that PGRN had no effects on the expression of NK cell surface molecules during NK cell expansion (Figure 1E).

In summary, recombinant PGRN suppresses NK cell expansion but did not affect the expression of NK cell surface markers.

Figure 1

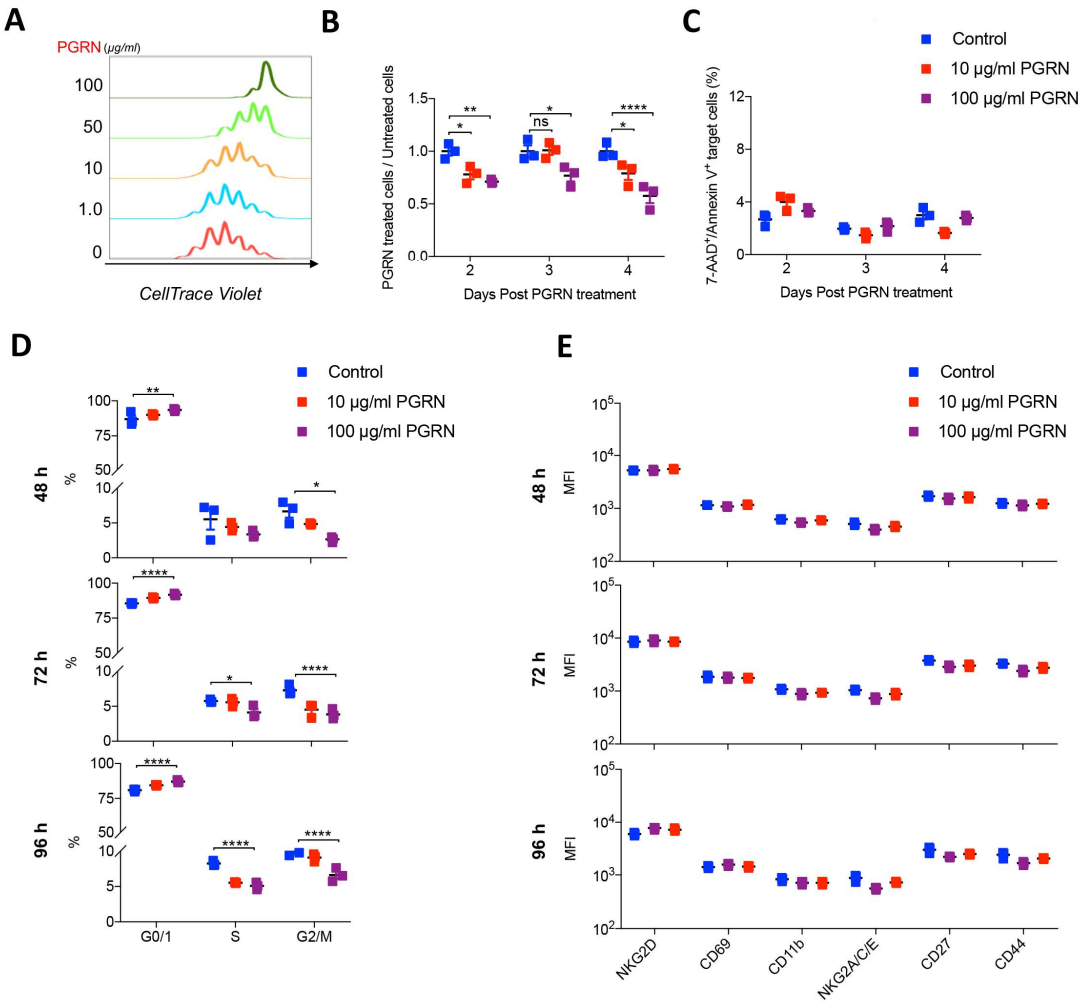


Figure 1. Recombinant PGRN suppresses murine NK cell expression in vitro.

(A) *NK cells isolated from mouse spleens were labeled by cell dye and then cultured with 1000 IU IL-2 and indicated doses of PGRN for 4 days. Flow cytometry analysis were performed in indicated time points;*

(B) *The same experiment setting to (A), the NK cell numbers were counted at indicated time points (n=3);*

(C) *The same experiment setting to (A); the apoptosis of NK cells was measured at indicated time points by flow cytometry analysis (n=3);*

(D) *The same experiment setting to (A); the cell cycle of NK cells was measured at indicated time points by flow cytometry analysis (n=3);*

(E) *The same experiment setting to (A); the levels of NK cell surface molecules were measured at indicated time points by flow cytometry analysis (n=3).*

*Data show mean ± s.e.m *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

4.2. PGRN limits NK-cell-mediated cytotoxicity

To investigate the effects of PGRN on NK cell function, NK-cell-based cytotoxicity assays were performed (Figure 2A), suggesting that PGRN treated NK cells remain lower cytotoxic activity against target RMA/s cells, a MHC-class-I-deficient cell line which is widely used for NK cell killing assays. Indeed, the transcription levels of NK cell Granzyme B and Perforin were reduced after PGRN treatment (Figure 2B). As expected, both the intracellular and secreted Granzyme B protein levels were suppressed by PGRN incubation (Figure 2C and 2D). In addition, we also observed reduced Granzyme B protein levels in expanded NK cells following PGRN treatments (Figure 2E).

On the basis of our in vitro observations, we stimulated NK cells harvested from splenocytes with the following cytokines: IL-2, IL-12, IL-15, and/or IL-18. Consistently, both the Granzyme B and IFN-γ levels were suppressed by treatments of recombinant PGRN (Figure 2F-2H). Proteolytic cleavage of PGRN by a neutrophil-derived elastase gives rise to smaller peptide fragments, termed as Granulins (561). We wondered whether Granulin would have similar effects on NK cells. Interestingly, the suppressive effects on NK cell Granzyme B expression disappeared following the treatments with elastase-digested PGRN (Figure 2I and 2J), suggesting that PGRN but not Granulins contains the suppressive effects on NK cell cytotoxicity.

Taken together, we conclude PGRN suppresses both NK cell expansion and NK cell cytotoxic activity.

Figure 2

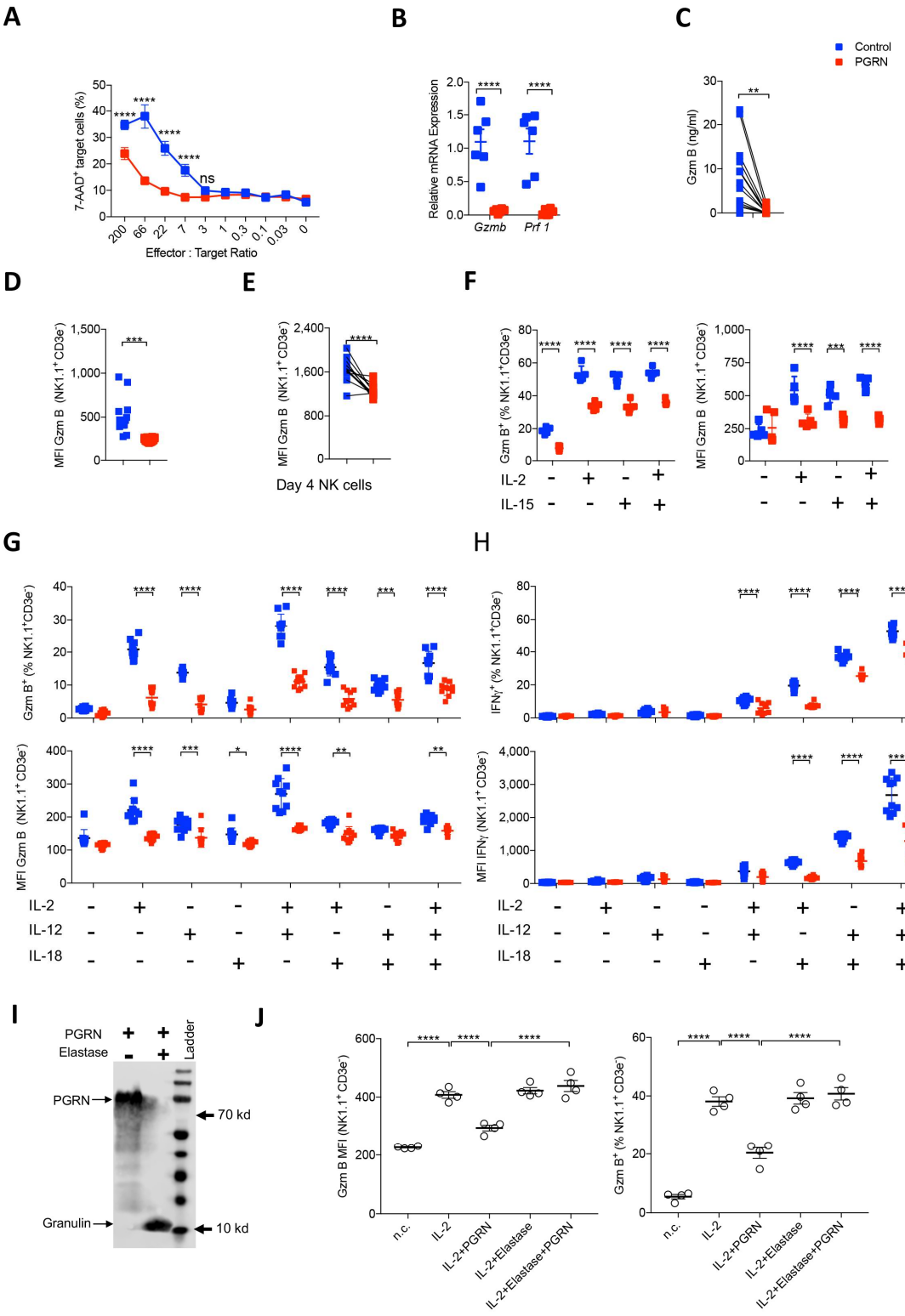

A
B
C
D
E
F
G
H
I
J
Control
PGRN
7-AAD+ target cells (%)
Effector : Target Ratio
Relative mRNA Expression
Gzmb
Prf 1
Gzm B (ng/ml)
MFI Gzm B (NK1.1+ CD3e-)
Day 4 NK cells
Gzm B+ (% NK1.1+CD3e-)
IFNγ+ (% NK1.1+CD3e-)
MFI IFNγ (NK1.1+ CD3e-)
IL-2
IL-12
IL-15
IL-18
PGRN
Elastase
Ladder
70 kd
10 kd
Granulin
Gzm B MFI (NK1.1+ CD3e-)
Gzm B+ (% NK1.1+CD3e-)
n.c.
IL-2
IL-2+PGRN
IL-2+Elastase
IL-2+Elastase+PGRN

Figure 2. Recombinant PGRN suppresses murine NK cell cytotoxicity in vitro.

(A) *NK cells isolated from mouse spleens were cultured with 1000 IU IL-2 and100 μg/ml PGRN for 4 days. These NK cells were then mixed with RMA/s cell at the indicated effector-to-target ratios for 18 hours. The cell viability of RMA/s cells was measured by flow cytometry with 7-AAD staining (n=5);*

(B) *Granzyme B and perforin mRNAs in PGRN treated NK cells were measured by q-PCR (n=6);*

(C) *The Granzyme B protein levels in supernatant from the PGRN treated NK cells were measured by ELISA (n=12);*

(D) *The intracellular Granzyme B protein levels in PGRN treated NK cells were measured by flow cytometry analysis (n=16);*

(E) *NK cells were firstly expanded by IL-2 for 4 days. Then these NK cells were subjected to PGRN treatment for 24 hours, the intracellular Granzyme B protein levels were measured by flow cytometry (n=12).*

(F) *NK cells from mouse splenocytes were activated with IL-2 and IL-15 with or without 25 μg/ml PGRN overnight. These cells were then subjected to flow cytometry analysis for Granzyme B expression in NK cells (n=5);*

(G) *NK cells from mouse splenocytes were incubated with IL-2, 12, 18 with or without 25 μg/ml PGRN for 6 hours. Flow cytometry analysis were performed for Granzyme B measurement (n=10);*

(H) *NK cells from mouse splenocytes were incubated with IL-2, 12, 18 with or without 25 μg/ml PGRN for 6 hours. Flow cytometry analysis were performed for IFN-γ measurement (n=10);*

(I) *Recombinant PGRN was digested with elastase. The efficiency of elastase disgestion was confirmed by western blot;*

(J) *NK cells from mouse splenocytes were activated with PGRN or the products from elastase treated PGRN. Flow cytometry analysis were performed for Granzyme B and IFN-γ measurement (n=4); Data show mean ± s.e.m *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

4.3. Recombinant PGRN limits human NK cell cytotoxicity

In order to extensively confirm the results that we obtained from the murine NK cell, we measured the effects of PGRN on human NK cells as well.

As expected, we observed a significant reduction in degranulation of total and $CD56^{dim}$ NK cells response to HLA-devoid cells K562 and 721.221 in the presence of PGRN (Figure 3A and 3B). Additionally, IFN-γ levels in human NK cells were also reduced following exposure to PGRN in response to K562 and 721.221 cells (Figure 3C and 3D).

In summary, these results indicate that recombinant PGRN suppresses human NK cell cytotoxic activity by limiting the expression of effector molecules as well.

Figure 3

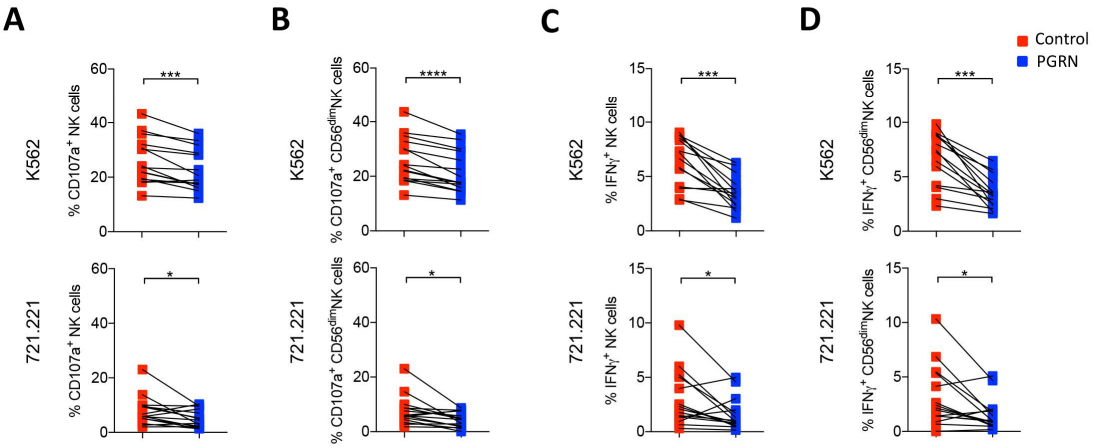


Figure 3. Recombinant PGRN suppresses human NK cell cytotoxic activity.

PBMCs from healthy donors were pretreated with 100 µg/ml overnight and then stimulated with K562 or 721.221 cell for 5 hours at an effector-to-target ratio at 1:10.

(A) *CD107a levels in total $CD56^{+}$ NK cells were measured by flow cytometry analysis (n=15);*

(B) *CD107a levels in $CD56^{dim}$ NK cells were measured by flow cytometry analysis (n=15);*

(C) *IFN-γ levels in total $CD56^{+}$ NK were measured by flow cytometry analysis (n=15);*

(D) *IFN-γ levels in $CD56^{dim}$ NK cells were measured by flow cytometry analysis (n=15);*

*Data show mean ± s.e.m *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

4.4. Perforin and TNF receptors are dispensable for PGRN-mediated NK cell suppression

Next, we explored the mechanism by which PGRN might limit NK cell cytotoxicity. NK cells can be self-regulated through cytotoxicity-mediated fratricide, which itself is triggered by the critical effector molecules perforin (561, 574).

Therefore, NK cells from *Prf1*$^{-/-}$ mice were used for *in vitro* expansion with different doses of PGRN. Interestingly, *Prf1*$^{-/-}$ NK cell expansion was still repressed by PGRN (Figure 4 A), suggesting that cytotoxicity-mediated fratricide was not the reason why NK cells were repressed by PGRN.

TNF receptors had been reported to be the receptors of PGRN (526). However, Granzyme B levels in NK cells from *Tnfrsf1a*$^{-/-}$ and *Tnfrsf1b*$^{-/-}$ mice were suppressed by PGRN during IL-2-induced NK cell activation (Figure 4B).

Taken together, these results indicate that NK cell fratricide and TNF receptors are dispensable for PGRN-mediated NK cell suppression.

Figure 4

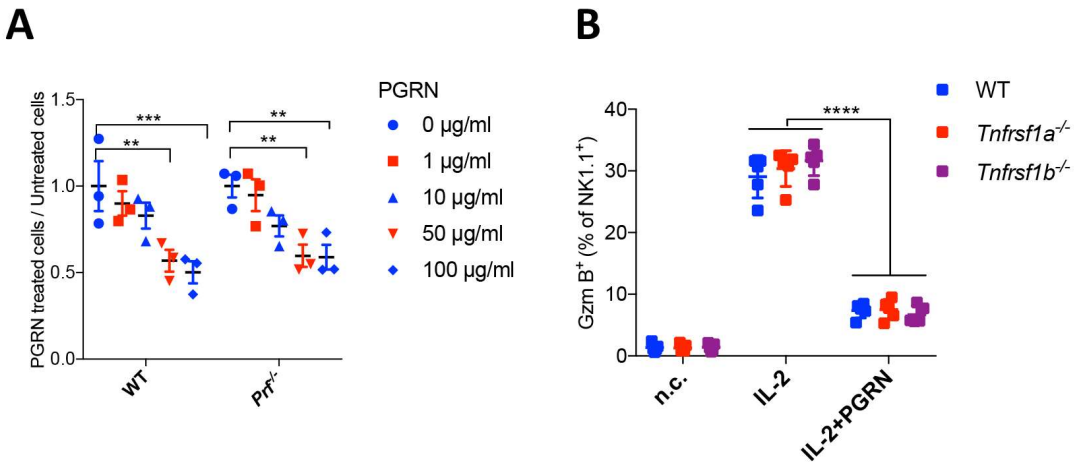


Figure 4. Perforin and TNF receptors are dispensable to PGRN-mediated NK cell suppression.

(A) *Freshly isolated NK cells from WT and Prf1$^{-/-}$ mice were treated with IL-2 and different doses of PGRN for 4 days (n=3). The NK cell numbers were measured at the end timepoint, Graphs illustrate a normalized value: (NK cell number in presence of PGRN) / (NK cell number in absence of PGRN);*

(B) *Splenocytes from Tnfrsf1a$^{-/-}$* and *Tnfrsf1b$^{-/-}$ mice were activated with IL-2 and 25µg/ml PGRN for 6 hours. The levels of Granzyme B in NK cells were measured by flow cytometry analysis (n=5);*

*Data show mean ± s.e.m *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

4.5. PGRN decreases Cyclin T1 and CDK9 expression in NK cells

We therefore wondered whether transcription factors regulating Granzyme B expression could be affected following PGRN treatment. It had been previously shown that PGRN could bind to the positive transcription elongation factor b (P-TEFb), which consists of Cyclin T1 and CDK9 (566). Knockdown of CDK9 and cyclin T1 results in the inhibition of T cell effector function attributable to the reduced expression of Perforin and Granzyme B (566). We wondered whether the same connection linking PGRN and CDK9/Cylclin T1 in T cells would also be applicable to NK cells.

Firstly, we treated NK cell cultures with SNS-032, a cyclin T1/CDK9 inhibitor, and found that Granzyme B expression in NK cell cultures was blocked (Figure 5A). We wondered whether PGRN was taken up by the NK cells. This was confirmed by using immunoblot analysis of lysates from PGRN treated NK cells (Figure 5B). Furthermore, in the presence of PGRN, there was a significant reduction of *Cdk9* mRNA levels in NK cells compared with the control conditions (Figure 5C), which was corroborated on a protein level using immunoblot analysis (Figure 5D). CDK9 cooperates with cyclin T1 to induce transcription (575), and we also found reduced protein levels of Cyclin T1 following treatment of NK cells with PGRN (Figure 3D). The reduction of cyclin T1 protein levels following incubation with PGRN was further confirmed by flow cytometry (Figure 5E) and immunofluorescence analyses (Figure 5F).

It has been previously reported that IL-2 promotes RNA polymerase II recruitment to the promoter of IL-2-induced genes (576). A histidine-rich domain in Cyclin T1 promotes phosphorylation of the C-terminal domain of RNA polymerase II (566, 577), which can be prevented by PGRN. Reduced phosphorylation (pSer2) of RNA polymerase II was indeed detected in NK cells during PGRN treatment (Figure 5G).

These above data indicate that PGRN limits the expression of key transcription factors CDK9 and cyclin T1, which are, in turn, critical for the transcription of NK cell effector molecules.

Figure 5

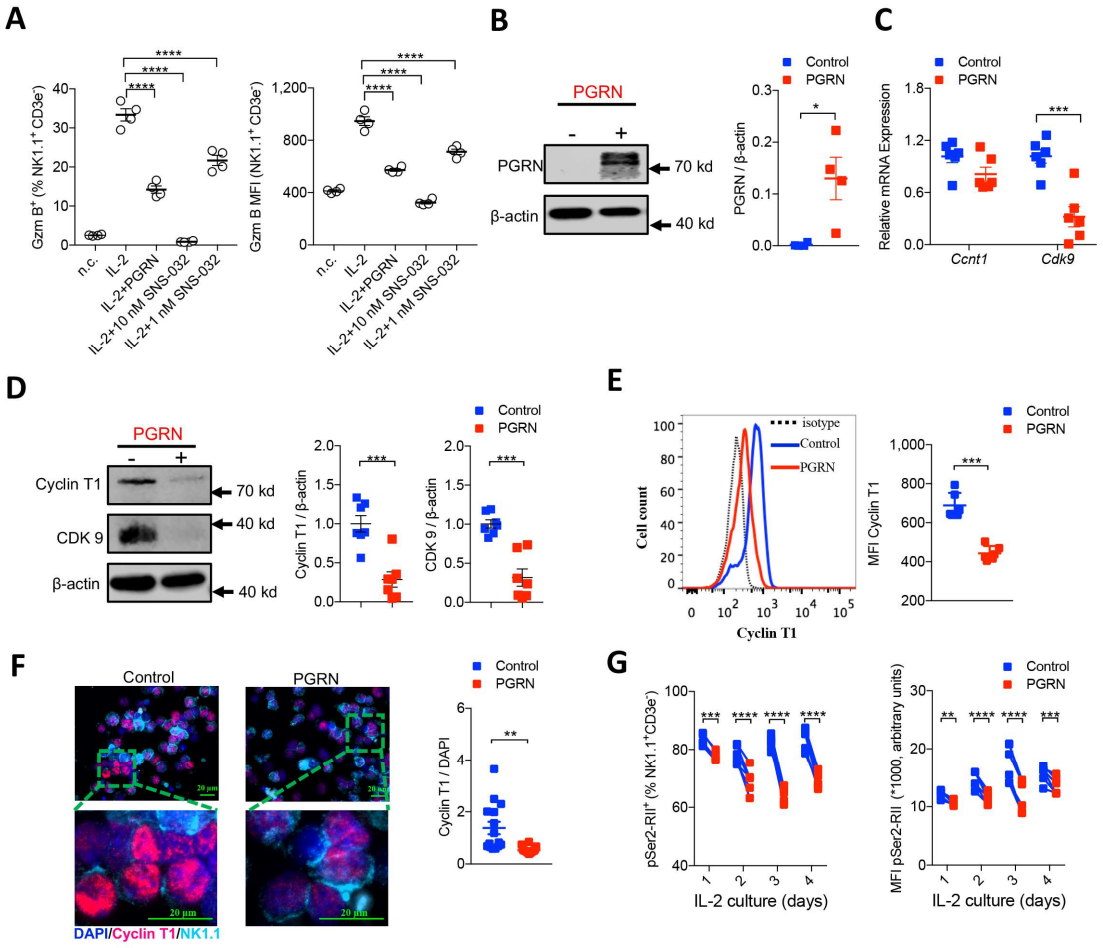


Figure 5. PGRN decreases Cyclin T1 and CDK9 expression to modulate NK cell cytotoxicity.

(A) *Splenocytes were treated with 1000 U/ml IL-2 and the Cyclin T1/CDK9 inhibitor SNS-032 for 6 hours (concentration as indicated). Granzyme B levels were measured by flow cytometry (n=4). The left panel represents the frequency of Granzyme B in NK cells, and the right panel represents the Granzyme B MFI in NK cells;*

(B) *The uptake of PGRN in NK cells were measured by western blot (n=4), the right panel represents the quantification of western blot data;*

(C) *Cyclin T1 and CDK9 mRNA levels were examined by q-PCR (n=6);*

(D) *Cyclin T1 and CDK9 protein levels were measured by western blotting (n=7), the middle and right panel represent Cyclin T1 and CDK9 quantification data;*

(E) *Cyclin T1 protein levels were measured by flow cytometry at day 3 after treatment with IL-2 and PGRN (n=6); the right panel represents the quantification of Cyclin T1 MFI;*

(F) *Cyclin T1 protein levels were examined by immunofluorescence (n=3), the right panel represents the quantification of Cyclin T1 by the ratio of Cyclin T1 to DAPI, Scale bars: 20μm;*

(G) *The phosphor-Ser2 RNA polymerase II frequency (pSer2-RII, upper panel) and MFI (lower panel) were measured by flow cytometry analysis at indicated time points after treatments with IL-2 and PGRN;*

*Data show mean ± s.e.m *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

4.6. IFN-I triggers mouse PGRN expression during LCMV infection

PGRN can be produced by macrophages, microglia, and endothelial cells in response to inflammatory stimuli and/or tissue damage (523). We wondered about the effects of PGRN during viral infection. To address this, we used the murine LCMV infection model, which is commonly used to study T cell-mediated immunity.

Firstly, PGRN mRNA levels were significantly induced by LCMV infections (Figure 6A). As expected, serum PGRN protein levels were elevated as well when compared with uninfected controls (Figure 6B). To investigate which cell types might be a source of PGRN, we analyzed serum PGRN levels in mice deficient for different immune subsets after LCMV infection. We did not find a reduction of PGRN levels in the absence of B cells and T cells (*Rag1*$^{-/-}$ mice), CD11c^{+} (CD11c-DTR), and CD169^{+} cells (CD169 diphtheria toxin receptor mice; CD169-DTR) after LCMV infection (Figure 6C-6E). However, serum PGRN levels were decreased following treatment with the phagocyte-depleting compound clodronate during LCMV infection (Figure 6F). As the reduction of PGRN was not complete compared to its basal levels, this indicates that cell types other than macrophages also contribute to PGRN secretion. Consistently, we observed comparable PGRN levels in lethally irradiated WT mice reconstituted with either control or *Grn*$^{-/-}$ bone marrow (BM) cells following LCMV infection (Figure 6G). This result indicates that cell subsets other than immune cells can also produce PGRN during LCMV infection.

Next, we wondered how PGRN secretion was triggered following infection. Early antiviral defense highly depends on the level of IFN-Is (356). Therefore, we speculated that innate IFN-I might trigger PGRN secretion. When BM-derived macrophages (BMDMs) were infected with LCMV in vitro, we observed increased secretion of PGRN in the supernatants (Figure 6H). As expected, in IFN-I binding receptor-deficient (*Ifnar1*$^{-/-}$) BMDMs, PGRN secretion was not

elevated and supernatant PGRN levels were similar to the naive conditions (Figure 4H). When *Ifnar1*$^{-/-}$ mice were infected with LCMV, we observed a reduction of *Grn* mRNA levels in spleen tissues when compared with WT control groups (Figure 6I). Furthermore, we observed no serum PGRN increase in *Ifnar1*$^{-/-}$ mice following infection of LCMV (Figure 6J), suggesting a key role of IFN-I in PGRN production.

Taken together, these data demonstrate a striking increase in PGRN secretion during LCMV infection, which was dependent on IFN-I signaling.

Figure 6

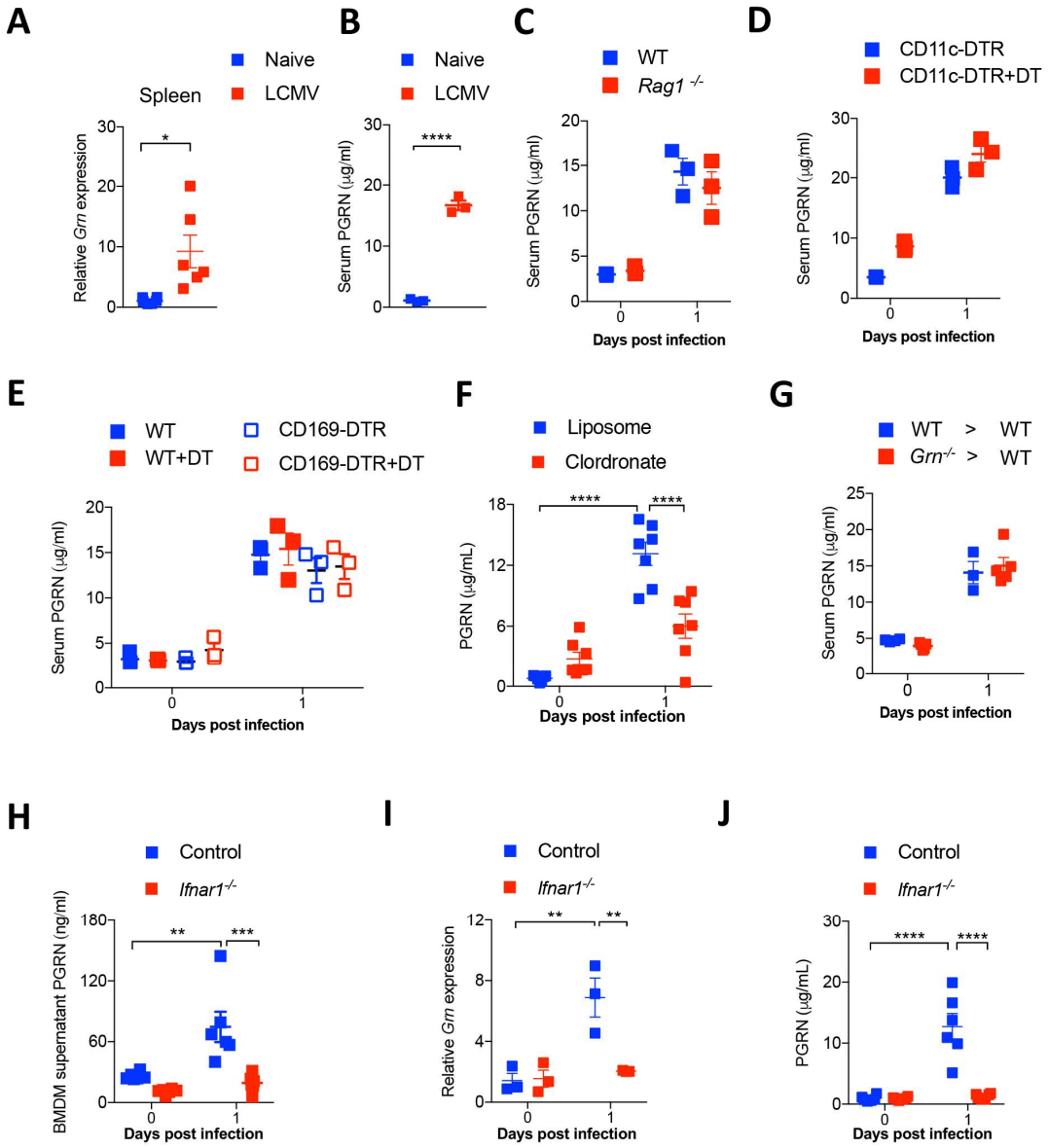


Figure 6. IFN-I triggers PGRN expression during viral infection.

*WT mice were infected with 2.0*10^6 pfu LCMV-WE.*

(A) *Grn mRNA levels in spleens were measured by q-PCR (n=6);*

(B) *Serum PGRN levels were measured by ELISA in naïve or virus infected (day 1) mice (n=6);*

(C) *WT and Rag1$^{-/-}$ mice were infected with 2*10^6 LCMV-WE. Serum PGRN levels were measured by ELISA at day 1 post infection (n=3);*

(D) *WT and CD169-DTR mice were injected with Diphtheria toxin (DT, 100 ng/mice) at day -2 and then these animals were subjected to LCMV-WE infection. Serum PGRN levels were measured by ELISA at day 1 post infection (n=3);*

(E) *WT and CD11c-DTR mice were injected with Diphtheria toxin (DT, 100 ng/mice) at day -2 and then these animals were subjected to LCMV-WE infection. Serum PGRN levels were measured by ELISA at day 1 post infection (n=3);*

(F) *Clodronate treated mice (day -1 injection) were infected with LCMV-WE. Serum PGRN levels were measured by ELISA at day 1 post infection (n=7);*

(G) *WT and Ifnar1$^{-/-}$ mice were infected with LCMV-WE. Serum PGRN levels were examined by ELISA at day 1 post infection (n=6);*

(H) *Grn mRNA levels in LCMV-WE infected WT and Ifnar1$^{-/-}$ spleens were examined by q-PCR (n=3);*

(I) *Serum PGRN levels in LCMV-WE infected WT and Ifnar1$^{-/-}$ mice were measured by ELISA (n=6);*

*Data show mean ± s.e.m *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

4.7. PGRN is dispensable for T cell development

It has been reported that PGRN may promote regulatory T cell development (578-580). This promoted us to ask whether PGRN governed the fate of other T cell subsets.

To address this question, the development of T cells in peripheral lymphoid organs was determined by flow cytometry. However, PGRN-deficient mice (*Grn*$^{-/-}$) displayed comparable CD8^{+} and CD4^{+} T cell differentiation when compared to PGRN competent mice (Figure 7A and 7B).

These results demonstrate that PGRN may not directly modulate T cell development.

Figure 7

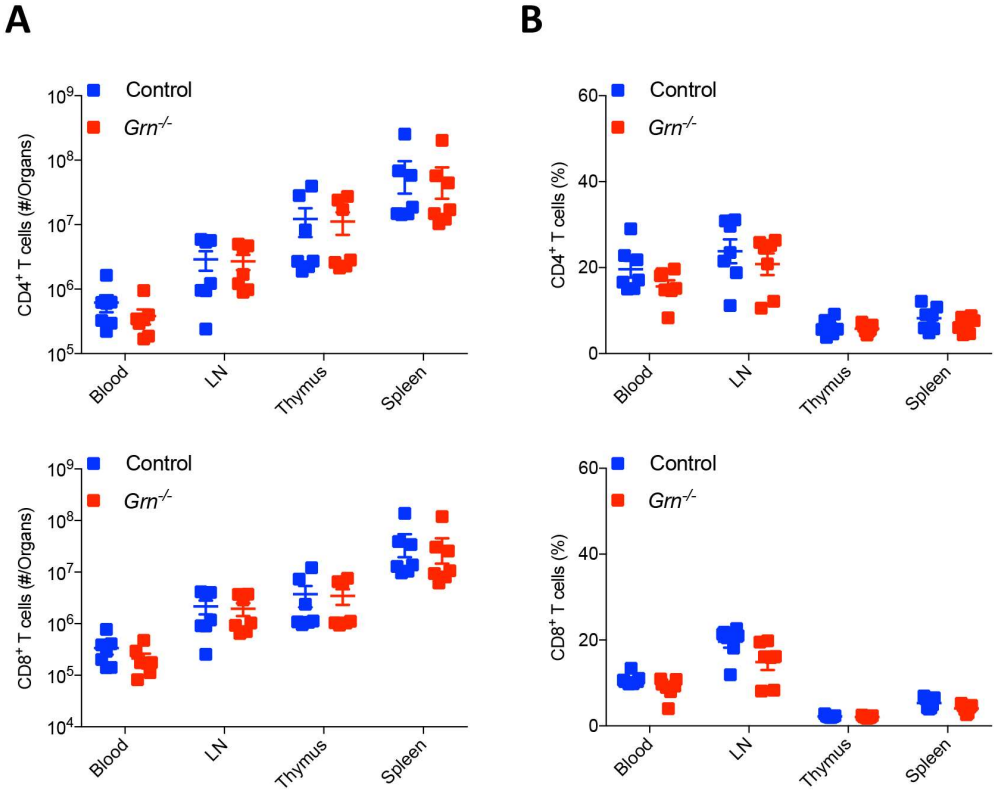


Figure 7. Naïve Grn$^{-/-}$ mice exhibit no gross T cell development phenotype.

(A) *CD4^{+} T cell number (upper panel) and CD8^{+} T cell number (lower panel) were measured by flow cytometry analysis in indicated tissues from WT or Grn$^{-/-}$ mice were (n=7);*

(B) *The frequency of CD4^{+} T cells (upper panel) and CD8^{+} T cells (lower panel) were measured in indicated tissues from WT or Grn$^{-/-}$ mice (n=7);*

*Data show mean ± s.e.m *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

4.8. T cell immunity is blunted by PGRN deficiency during LCMV infection

To address whether PGRN regulate T cell function during viral infections, antiviral T cell responses were determined in WT and *Grn*$^{-/-}$ mice following LCMV-WE infection.

Strikingly, *Grn*$^{-/-}$ mice showed a reduced presence of antiviral CD8^{+} T cells when compared with corresponding control animals (Figure 8A). Consistently, we observed increased expression of PD-1 on antiviral (gp33-H2-Db-tetramer^{+}) T cells from *Grn*$^{-/-}$ mice, which is associated with T cell exhaustion (Figure 8B). Furthermore, when we restimulated splenocytes with LCMV gp33 peptides, we observed reduced IFN-γ^{+}CD8^{+} T cells from *Grn*$^{-/-}$ mice compared with controls (Figure 8C). Control and clearance of LCMV depends on antiviral CD8^{+} T cell immunity, and as expected, increased LCMV titers were detected in *Grn*$^{-/-}$ mice (Figure 8D), suggesting the dysfunction of antiviral immunity in *Grn*$^{-/-}$ mice.

In addition, the levels of LCMV nuclear protein-positive (LCMV-NP^{+}) cells in sections of snap-frozen liver tissue harvested from *Grn*$^{-/-}$ mice were increased when compared with control animals (Figure 8E). Hepatic replication of LCMV can result in T cell-mediated liver damage (581). The enzyme alanine aminotransferase (ALT) activity in the serum of LCMV-infected *Grn*$^{-/-}$ animals was markedly increased compared with control animals, suggesting that liver pathology was increased in absence of PGRN (Figure 8F). In addition, when we treated WT mice with recombinant PGRN throughout LCMV infection, we observed enhanced antiviral CD8^{+} T cell immunity (Figure 8G–8I).

Taken together, we conclude that PGRN deficiency represses anti-viral CD8^{+} T cell immunity, resulting in virus persistence in *Grn*$^{-/-}$ mice.

Figure 8

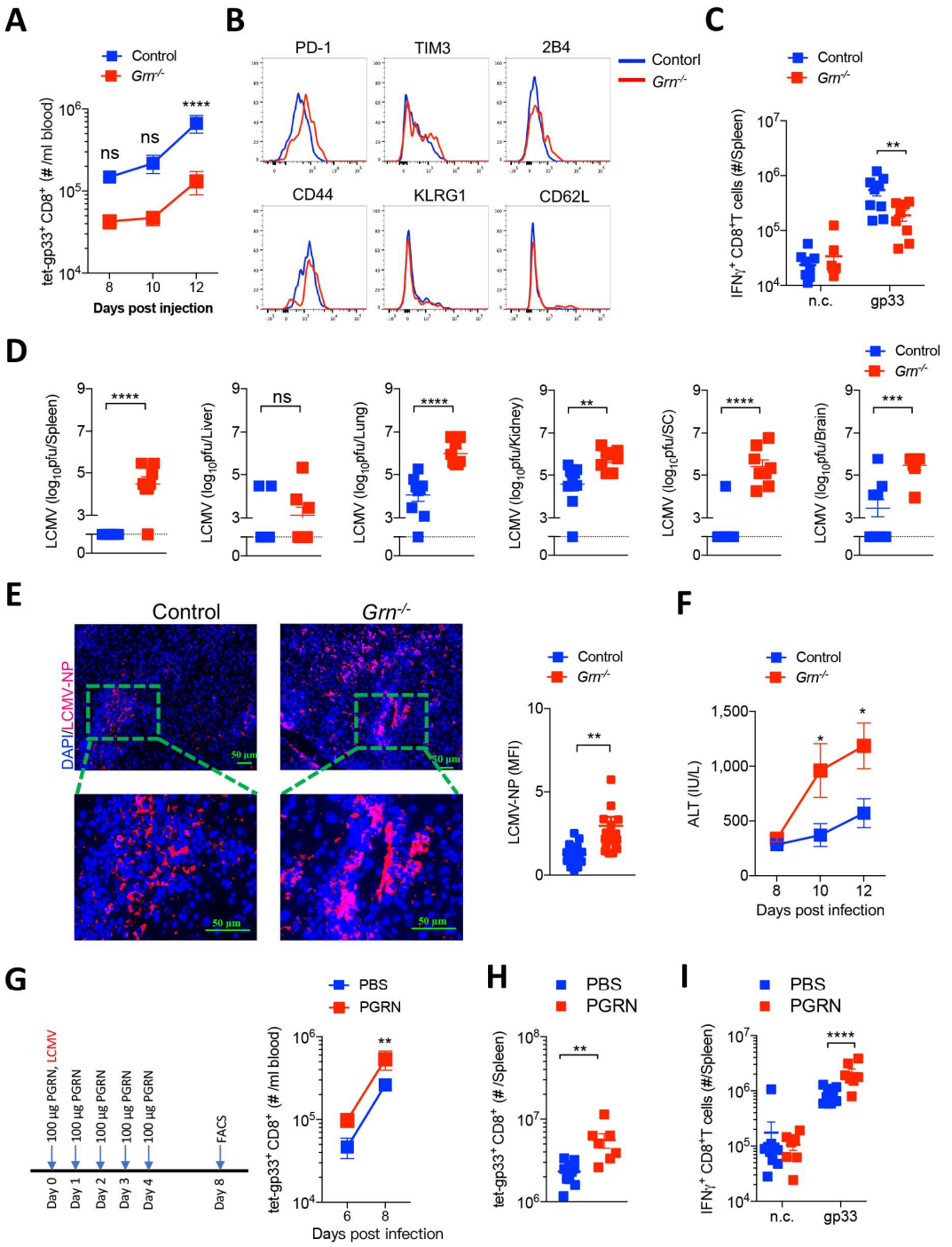

A
Control
Grn-/-
tet-gp33+ CD8+ (# /ml blood)
ns
ns
****
Days post injection
B
PD-1
TIM3
2B4
CD44
KLRG1
CD62L
Contorl
Grn-/-
C
Control
Grn-/-
IFNγ+ CD8+T cells (#/Spleen)
**
n.c.
gp33
D
Control
Grn-/-
LCMV (log10pfu/Spleen)
LCMV (log10pfu/Liver)
LCMV (log10pfu/Lung)
LCMV (log10pfu/Kidney)
LCMV (log10pfu/SC)
LCMV (log10pfu/Brain)
E
Control
Grn-/-
DAPI/LCMV-NP
50 μm
LCMV-NP (MFI)
F
ALT (IU/L)
Days post infection
G
100 μg PGRN, LCMV
100 μg PGRN
Day 0
Day 1
Day 2
Day 3
Day 4
Day 8
FACS
PBS
PGRN
tet-gp33+ CD8+ (# /ml blood)
Days post infection
H
tet-gp33+ CD8+ (# /Spleen)
I
IFNγ+ CD8+T cells (#/Spleen)

Figure 8. LCMV virus persists in Grn$^{-/-}$ mice by impaired anti-viral CD8^{+} T cell immunity.

*WT and Grn$^{-/-}$ mice were infected with 2*10^6 pfu LCMV-WE.*

(A) *Absolute numbers of antiviral CD8^{+} T cells in the blood were determined by flow cytometry analysis at the indicated time points during LCMV-WE infection (n=7-10);*

(B) *T cell exhaustion levels on antiviral CD8^{+} T cells were measured by flow cytometry;*

(C) *At the end time point, the splenocytes were re-stimulated with LCMV-gp33 peptides for 6 hours, IFN-γ^{+} CD8^{+} T cells were determined by flow cytometry (n=7-10), n.c. indicates no gp33 stimulation group;*

(D) *Plaque assays were performed for measuring LCMV-WE virus titer in indicated organs at day 12 post infection (n=7-10);*

(E) *Sections of snap-frozen liver tissues from WT and Grn$^{-/-}$ mice were analyzed for LCMV-NP levels (n=6), the right panel represents the quantification of LCMV-NP^{+} signals, scale bars: 50μm;*

(F) *The activity of ALT in WT and Grn$^{-/-}$ serum was measured at the indicated time points after infection (n=6);*

(G) *The schedule of the administration of recombinant PGRN in WT mice and the blood anti-viral CD8^{+} T cell responses in indicated time points are determined (n=7-10);*

(H) *The absolute numbers of antiviral CD8^{+} T cells in spleen tissues is shown (n=7-10);*

(I) *IFN-γ^{+} CD8^{+} T cells were determined by flow cytometry at day 8 post infection (n=7-10), n.c. indicates no adding of peptides;*

*Data show mean ± s.e.m *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

4.9. LCMV replication remains intact in presence of PGRN

Since the virus persists in mice, we speculated that PGRN might exert direct antiviral effects.

The replications of LCMV virus were performed with or without PGRN in different cell lines. However, when N2a neuroblastoma, Vero, or L929 cells were exposed to different dose of PGRN, we did not observe any correlations between PGRN and LCMV replication (Figure 9A-9C).

These results indicated that PGRN may have no impacts on LCMV replication in vitro.

Figure 9

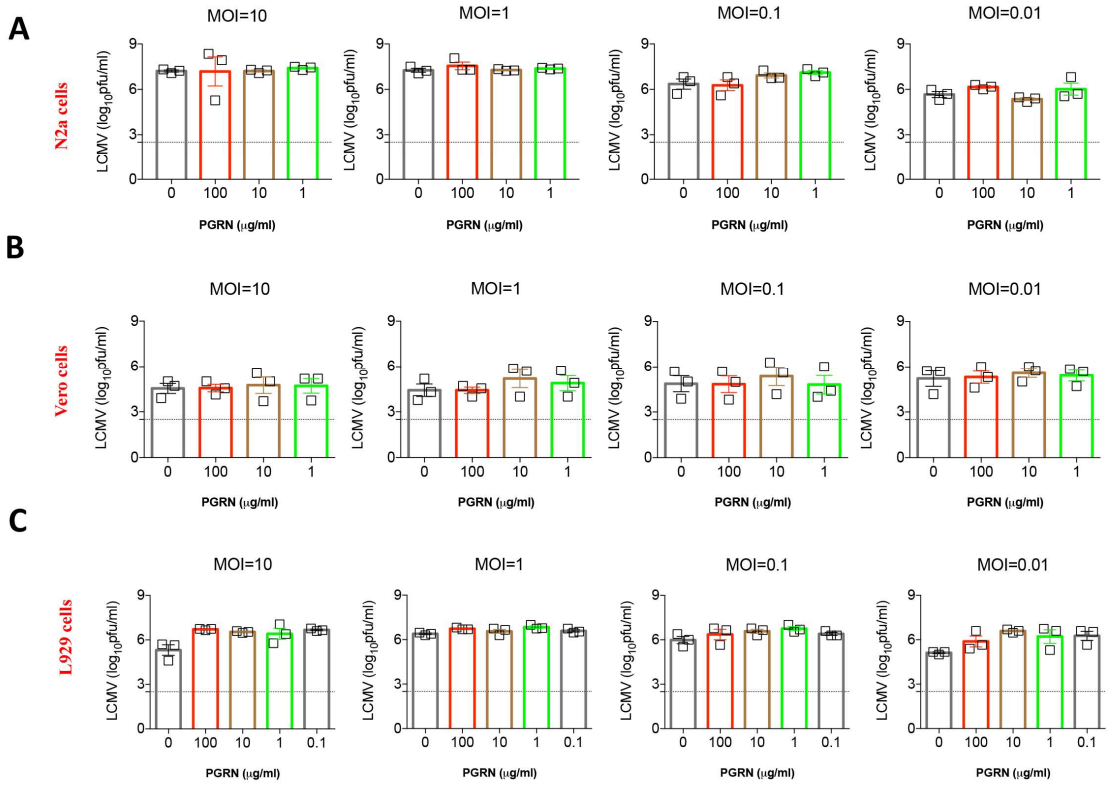


Figure 9. ***LCMV virus persists in Grn$^{-/-}$ mice by impairing anti-viral CD8^{+} T cell immunity.***

(A) *N2a neuroblastoma cells were cultured with indicated dose of PGRN and infected with indicated dose of LCMV-WE. The virus titer in the supernatants was determined by plaque assays (n=3);*

(B) *Vero cells were cultured with indicated dose of PGRN and infected with the indicated dose of LCMV-WE. The virus titer in the supernatants were determined by plaque assays (n=3);*

(C) *L929 cells were cultured with an indicated dose of PGRN and infected with a indicated dose of LCMV-WE. The virus titer in the supernatants was determined by plaque assays (n=3);*

*Data show mean ± s.e.m *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

4.10. PGRN extrinsically regulates anti-viral T cell immunity

Next, we examined whether the observed defective T cell immunity following LCMV infection in *Grn*$^{-/-}$ mice was caused by T cell intrinsic effects. We adoptively transferred negatively sorted CD8^{+} T cells, from a mouse carrying a TCR recognizing the LCMV antigen gp33 (P14 cells) (582), to *Grn*$^{-/-}$ and control mice. We found reduced expansion of P14 cells in *Grn*$^{-/-}$ hosts following LCMV infection when compared with control hosts (Figure 10A). Moreover, LCMV virus still persists in *Grn*$^{-/-}$ hosts even after P14 cells were transferred, whereas virus in WT hosts was eliminated (Figure 10B).

ALT and aspartate aminotransferase (AST) activities keep in higher levels in *Grn*$^{-/-}$ hosts than control hosts (Figure 10C). Furthermore, upregulation of α-SMA, a liver fibrosis marker, in *Grn*$^{-/-}$ liver tissue sections were observed, when compared with control mice (Figure 10D).

These results demonstrated that PGRN deficiency might extrinsically suppress antiviral CD8^{+} T cell responses that result in the persistence of LCMV.

Figure 10

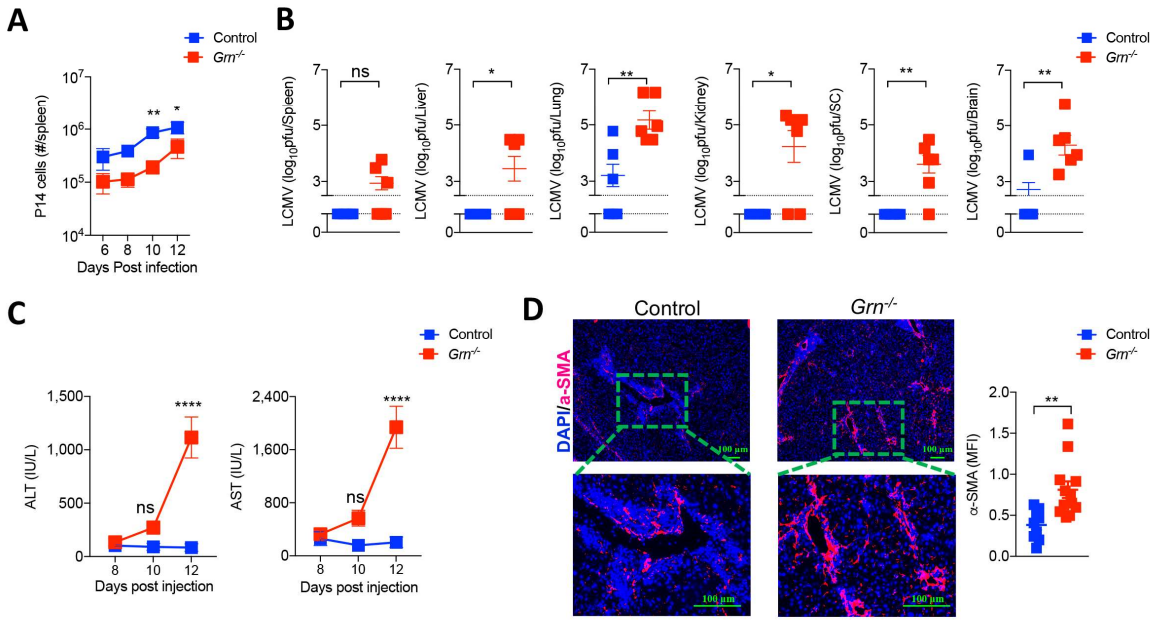


Figure 10. PGRN extrinsically regulates antiviral CD8$^+$ T cell immunity in LCMV infection.

3000 negatively sorted P14 cells (TCR transgenic T cell recognizing LCMV-gp33 epitope) were transferred into WT and Grn$^{-/-}$ mice and then following LCMV-WE infection of the mice.

(A) *The absolute numbers of P14$^+$ cells in blood was measured at the indicated time points after infection (n=6);*

(B) *Plaque assays were performed for testing the virus titer in indicated organs (n=6);*

(C) *Serum ALT and AST activities were examined at the indicated time points after infection (n=6), the right panel represents AST activity, the left panel represents ALT activity;*

(D) *Sections of snap-frozen liver tissues (day 12 post infection) were stained with α-SMA antibodies (n=3), right panel indicates quantification of fluorescence intensity, scale bars: 100 μm;*

*Data show mean ± s.e.m *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

4.11. PGRN is dispensable for T cell activation in vitro

Next, we wondered whether PGRN affected T cell activation. Stimulation of WT $CD8^+$ T cells with anti-CD3/anti-CD28 antibodies or PMA/ionomycin in the presence or absence of PGRN did not affect proliferation or activation of $CD8^+$ T cells (Figure 11A and 11B). To further investigate the effects of PGRN on T cells, purified $CD8^+$ T cells were activated with anti-CD3/anti-CD28 antibodies and recombinant PGRN for 24 hours. Flow cytometry analysis showed that PGRN had no effects on apoptosis of $CD8^+$ T cells (Figure 11C). Additionally, PGRN also had no effects on IFN-γ expression of $CD8^+$ T cells in the absence or presence of PGRN, when splenocytes from LCMV-infected mice were rechallenged with gp33 peptides (Figure 11D).

These results indicate that PGRN may not directly regulate T cell expansion and activation.

Figure 11

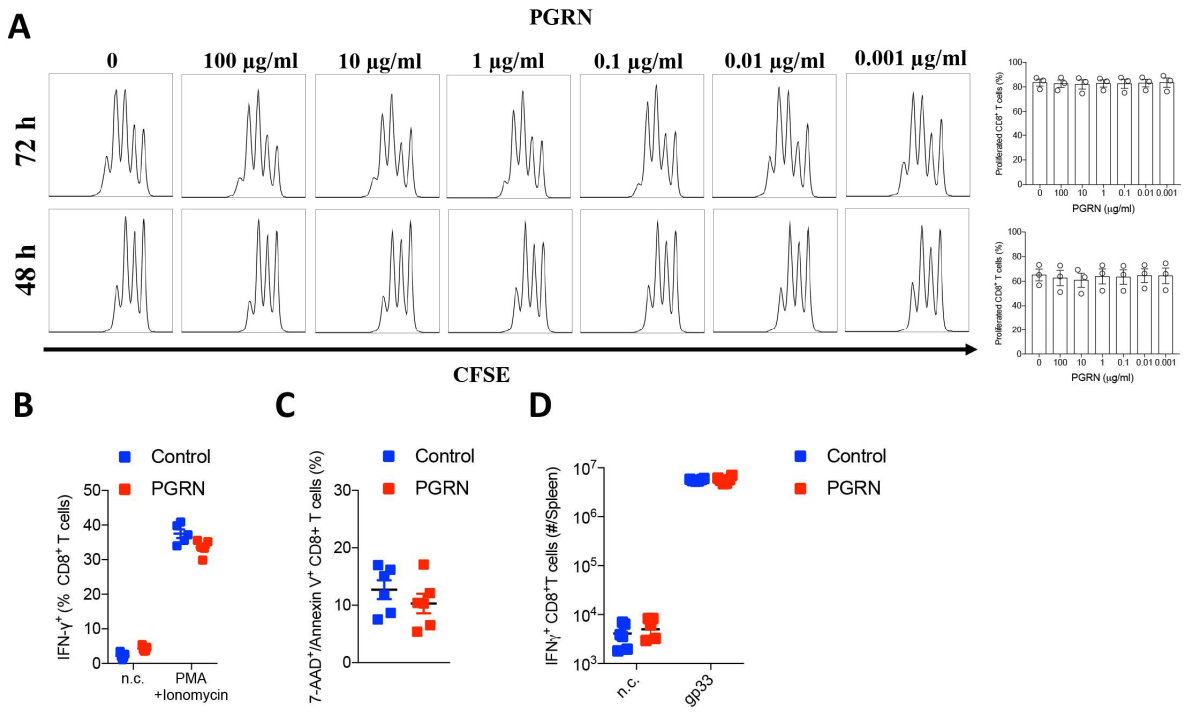


Figure 11. PGRN has no effects on T cell activation and proliferation.

(A) *Purified $CD8^+$ T cells were labeled with CFSE and then stimulated with anti-CD3/CD28 in the presence of indicated doses of PGRN in vitro. Flow cytometry was performed at the indicated time points (n=3);*

(B) *Splenocytes from WT mice were stimulated with PMA/Ionomycin for 6 hours. IFN-γ^+ cells were determined by flow cytometry (n=5);*

(C) *Naïve $CD8^+$ T cells were activated with anti-CD3/CD28 antibodies for 24 hours with PGRN. The cell apoptosis was determined by flow cytometry (n=6);*

(D) *Splenocytes from LCMV infected WT mice were restimulated with gp-33 peptides and PGRN for 6 hours, the IFN-γ^+ $CD8^+$ cells were determined by flow cytometry (n=6);*

*Data show mean ± s.e.m *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

4.12. PGRN is dispensable for NK cell development

While PGRN had a direct NK cell inhibitory activity in vitro, absence of PGRN in vivo resulted in reducing T cell activity following infection. We therefore speculated that NK cells in *Grn*$^{-/-}$ mice limited antiviral T cells during LCMV infection, which could contribute to establishment of a persistent viral infection (497-499).

We first examined the development of NK cell and innate lymphoid cells (ILCs) in naive animals. *Grn*$^{-/-}$ mice had the same amount of the NK cell precursors as control mice (Figure 12A). Absolute numbers of both NK cell and ILC subsets were comparable between naive control and naïve *Grn*$^{-/-}$ mice (Figure 12B and 12C). The surface receptor expression on NK cells from different organs was also similar in both groups before infection (Figure 12D).

Taken together, the development of NK cells may not be affected in *Grn*$^{-/-}$ mice.

Figure 12

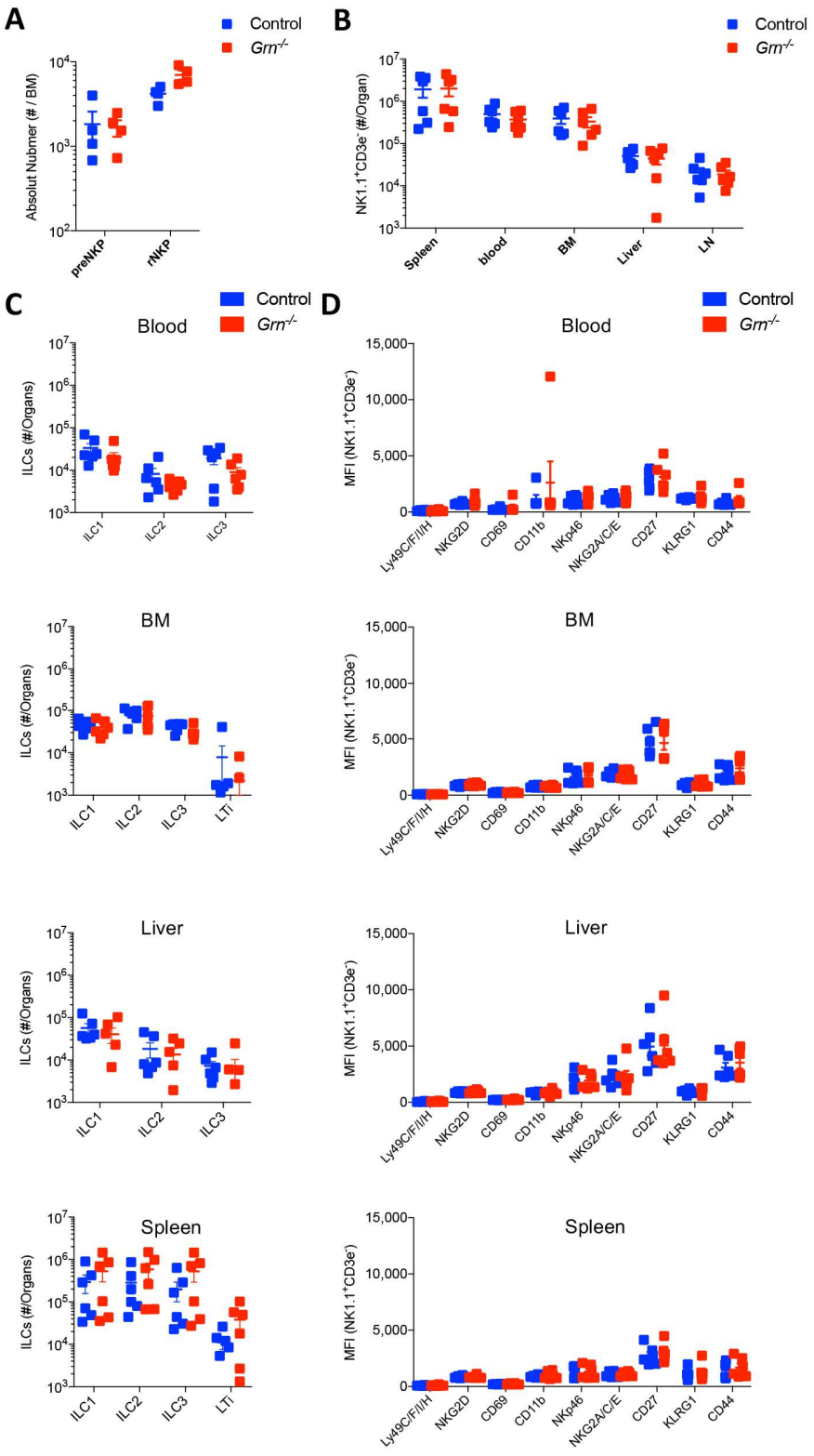

A
Control
Grn-/-
Absolut Nubmer (# / BM)
preNKP
rNKP
B
Control
Grn-/-
NK1.1+CD3e- (#/Organ)
Spleen
blood
BM
Liver
LN
C
Control
Grn-/-
Blood
ILCs (#/Organs)
ILC1
ILC2
ILC3
D
Control
Grn-/-
Blood
MFI (NK1.1+CD3e-)
Ly49C/F/I/H
NKG2D
CD69
CD11b
NKp46
NKG2A/C/E
CD27
KLRG1
CD44
BM
ILCs (#/Organs)
ILC1
ILC2
ILC3
LTi
BM
MFI (NK1.1+CD3e-)
Liver
ILCs (#/Organs)
Liver
MFI (NK1.1+CD3e-)
Spleen
ILCs (#/Organs)
Spleen
MFI (NK1.1+CD3e-)

Figure 12. PGRN is dispensable for NK cell differentiation.

(A) *Absolute numbers of NK cell progenitor preNKP ($Lin^-2B4^+CD27^+CD127^+CD122^-Flt3^-$) and rNKP ($Lin^-2B4^+CD27^+CD127^+CD122^+Flt3^-$) were measured by flow cytometry in bone marrow (BM) samples from control and $Grn^{-/-}$ mice (n=3);*

(B) *$CD3e^-NK1.1^+$ cells in indicated organs were examined in $Grn^{-/-}$ and control mice (n=6);*

(C) *ILC1 ($Lin^-NK1.1^+ROR\gamma T^-Eomes^-$), ILC2 ($Lin^-NK1.1^-ROR\gamma T^-CD11b^-GATA\text{-}3^+$), ILC3 ($Lin^-ROR\gamma T^+CD4^-$) and LTi ($Lin^-ROR\gamma T^+CD4^+$) subsets were measured by flow cytometry in organs harvested from naive control and $Grn^{-/-}$ mice as indicated. Gating strategy excluded dead and Lin (CD3, CD5, CD8, CD19, Ly-6G, TCR, and FcγR1) cells (n=6);*

(D) *Inhibitory and activating receptors of $CD3e^-NK1.1^+$ cells in the blood and peripheral lymphoid organs as indicated were examined in naive control and $Grn^{-/-}$ mice (n=6);*

*Data show mean ± s.e.m *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

4.13. PGRN is dispensable for NK cell surface receptors expression

In addition, NK cell numbers and surface NK cell receptor expression changed in a similar way in control and *Grn*$^{-/-}$ mice following LCMV infection (Figure 13A and 13B). NK cells from *Grn*$^{-/-}$ and control splenocytes showed comparable Granzyme B and IFN-γ production following NK cell stimulation during cytokine-induced activation (Figure 13C and 13D). These data suggest that NK cell development was not dependent on PGRN.

As PGRN can be produced in vitro by macrophages following LCMV infection, we mixed naive WT NK cells with LCMV-infected BMDMs from control and *Grn*$^{-/-}$ mice. CD27^{+}CD11b^{+} NK cells have been shown to exhibit highly cytotoxic effects to RMA/s cells, whereas CD27^{-} CD11b^{+} NK cells only displayed background levels of cytotoxicity in this setting (447). Following exposure of LCMV-infected *Grn*$^{-/-}$ BMDMs, we observed an increased presence of CD27^{+}CD11b^{+} NK cells compared with control BMDMs (Figure 13E).

Figure 13

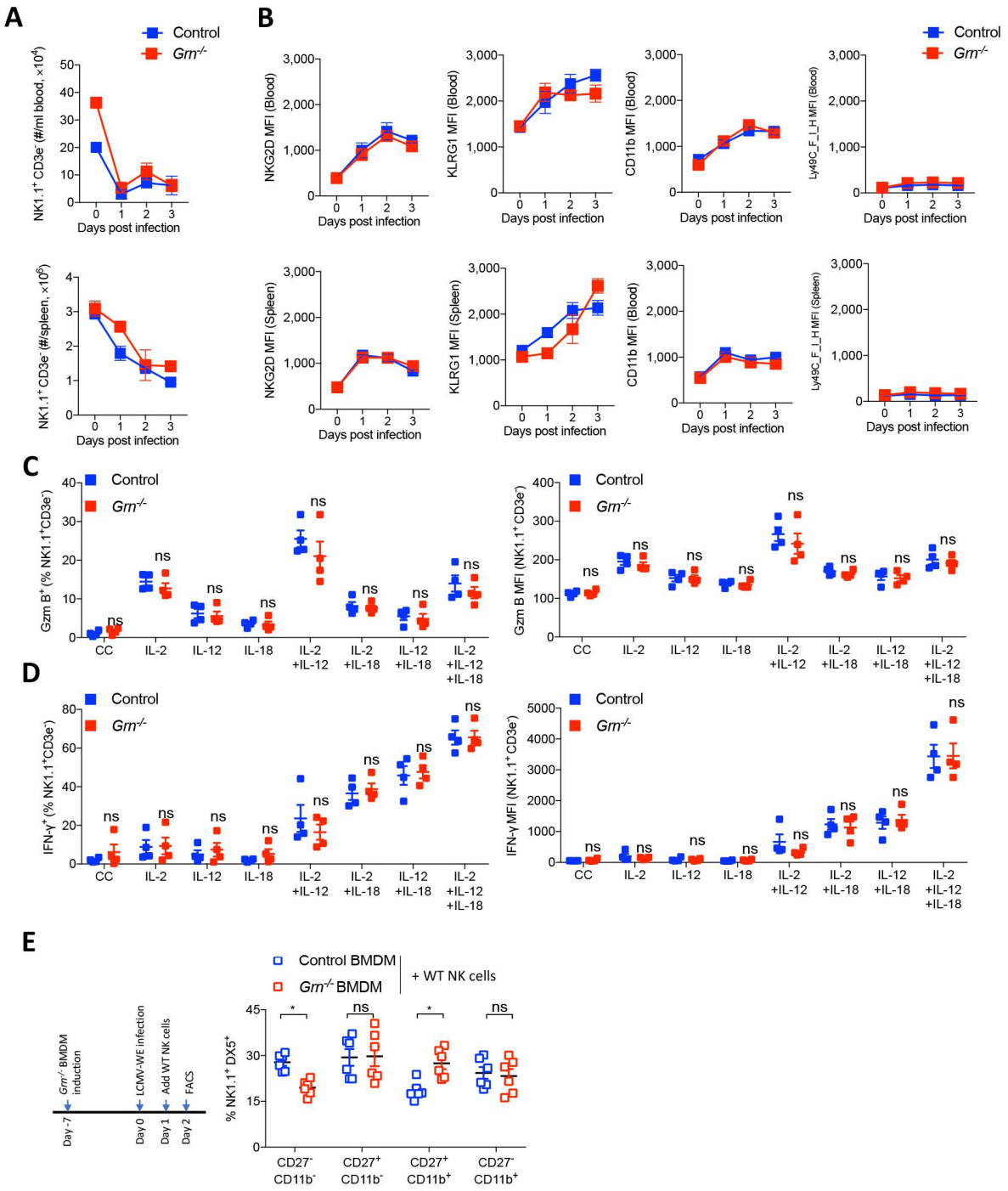

A
B
C
D
E
Control
Grm-/-
NK1.1+ CD3e- (#/ml blood, x10^4)
NK1.1+ CD3e- (#spleen, x10^6)
Days post infection
NKG2D MFI (Blood)
KLRG1 MFI (Blood)
CD11b MFI (Blood)
Ly49C_F_I_H MFI (Blood)
NKG2D MFI (Spleen)
KLRG1 MFI (Spleen)
CD11b MFI (Blood)
Ly49C_F_I_H MFI (Spleen)
Gzm B+ (% NK1.1+CD3e-)
Gzm B MFI (NK1.1+ CD3e-)
IFN-γ+ (% NK1.1+CD3e-)
IFN-γ MFI (NK1.1+ CD3e-)
CC
IL-2
IL-12
IL-18
IL-2 +IL-12
IL-2 +IL-18
IL-12 +IL-18
IL-2 +IL-12 +IL-18
ns
Control BMDM
Grm-/- BMDM
+ WT NK cells
Grm-/- BMDM induction
LCMV-WE infection
Add WT NK cells
FACS
Day -7
Day 0
Day 1
Day 2
% NK1.1+ DX5+
CD27- CD11b-
CD27+ CD11b-
CD27+ CD11b+
CD27- CD11b+

Figure 13. PGRN is dispensable for NK cell functional receptors expression during LCMV infection.

(A) *Control and $Grn^{-/-}$ mice were infected with LCMV-WE. Absolute numbers of NK cells in indicated organs were determined by flow cytometry (n=4);*

(B) *NK cell surface receptors were measured by flow cytometry (n=4);*

(C) *$Grn^{-/-}$ splenocytes were activated with different cytokines for 6 hours. Granzyme B^{+} NK cells were measured by flow cytometry (n=4);*

(D) *$Grn^{-/-}$ splenocytes were activated with different cytokines for 6 hours. IFN-γ^{+} NK cells were measured by flow cytometry (n=4);*

(E) *BMDM cells were infected with LCMV for 24 hours and isolated naïve purified WT NK cells were added to the cultures for another 24 hours. The maturation of these NK cell was measured by flow cytometry (n=6);*

*Data show mean ± s.e.m *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

4.14. PGRN deficiency results in excessive NK cell-mediated regulation of antiviral T cell responses

Although the surface receptors expression on NK cells in *Grn*$^{-/-}$ mice is comparable to WT NK cells, the cytotoxic activity of these NK cells remains unknown. To address this question, we next measured the cytotoxic activity of NK cells during LCMV infection.

Interestingly, re-challenge of LCMV-infected splenic NK cells with RMA/s cells induced more IFN-γ^+ NK cells in the *Grn*$^{-/-}$ group (Figure 14A). Notably, NK cell-mediated killing was enhanced as well in ex vivo assays using splenocytes harvested from LCMV-infected *Grn*$^{-/-}$ mice compared with controls (Figure 14B). Consistently, NK cell mediated in vivo anti-viral T cell lysis was enhanced in *Grn*$^{-/-}$ mice concluded by the more severe apoptotic phenotype of P14$^+$ cells in *Grn*$^{-/-}$ mice at day 1 after infection than that in WT hosts (Figure 14C).

As expected, antiviral CD8$^+$ T cell immunity in *Grn*$^{-/-}$ mice was restored when we depleted NK cells prior to LCMV infection (Figure 14D). Consequently, NK cell-depleted *Grn*$^{-/-}$ mice had more splenic CD8$^+$ IFN-γ^+ T cells following gp33 re-stimulation than that in WT mice (Figure 14E). In addition, virus was eliminated at day 12 after infection following NK cell depletion in *Grn*$^{-/-}$ mice, in sharp contrast with NK cell-competent *Grn*$^{-/-}$ mice, which showed high LCMV titers (Figure 14F). Furthermore, when we analyzed LCMV-NP expression in liver tissue, we found enhanced LCMV-NP expression in *Grn*$^{-/-}$ mice but reduced expression in NK cell-depleted *Grn*$^{-/-}$ mice (Figure 7G). At this time point, liver enzymes (AST/ALT; Figure 14H), as well as α-SMA expression (Figure 14I), was markedly reduced in the absence of NK cells in *Grn*$^{-/-}$ animals.

Taken together, we conclude that PGRN reduces cytotoxic NK cell activity and, as a consequence, increases antiviral T cell immunity, resulting in clearance of LCMV.

Figure 14

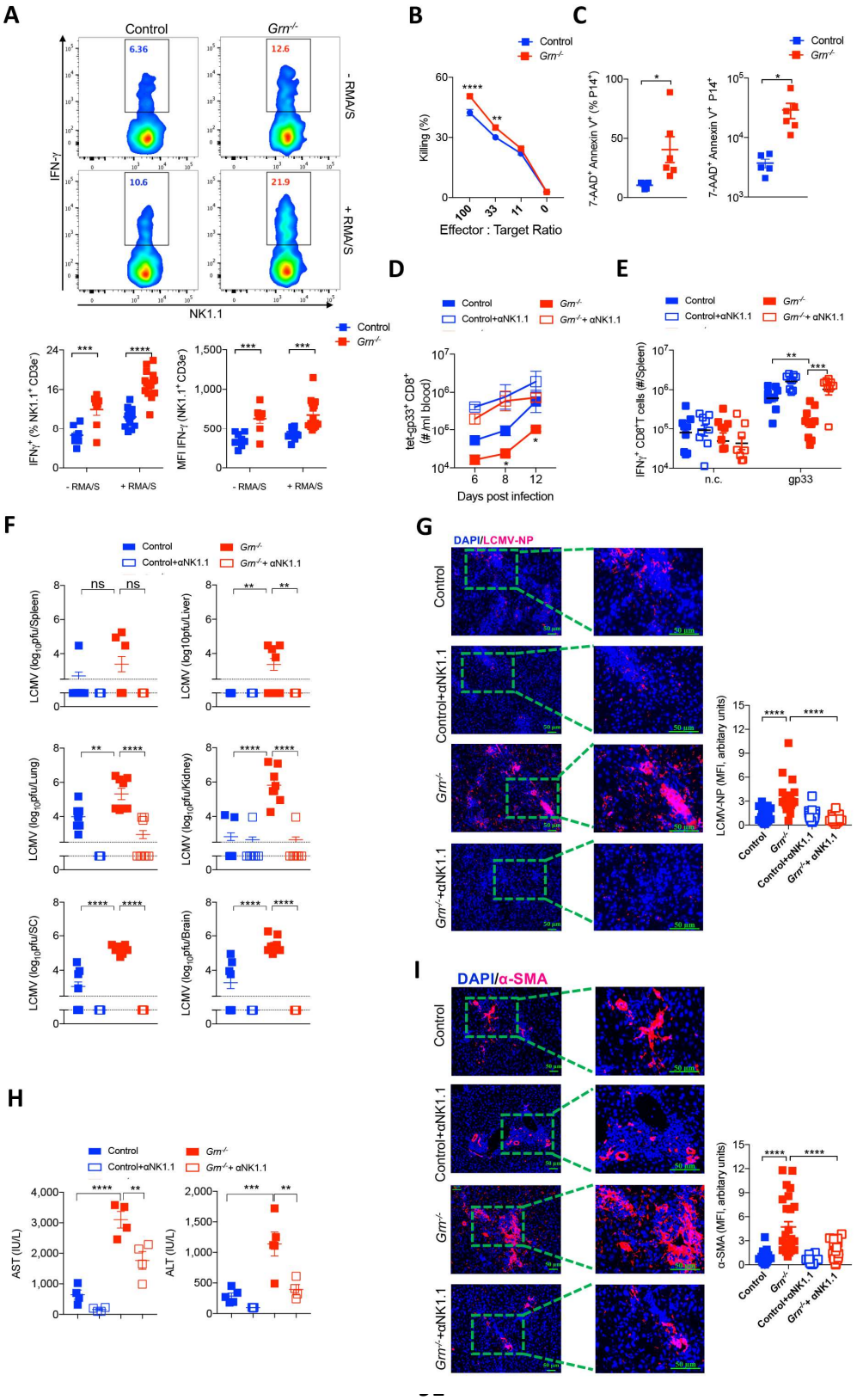

A
Control
Grn-/-
6.36
12.6
10.6
21.9
- RMA/S
+ RMA/S
IFN-γ
NK1.1
Control
Grn-/-
IFNγ+ (% NK1.1+ CD3e-)
MFI IFN-γ (NK1.1+ CD3e-)
- RMA/S
+ RMA/S
B
Control
Grn-/-
Killing (%)
Effector : Target Ratio
C
Control
Grn-/-
7-AAD+ Annexin V+ (% P14+)
7-AAD+ Annexin V+ P14+
D
Control
Control+αNK1.1
Grn-/-
Grn-/-+ αNK1.1
tet-gp33+ CD8+ (#/ml blood)
Days post infection
E
Control
Control+αNK1.1
Grn-/-
Grn-/-+ αNK1.1
IFNγ+ CD8+T cells (#/Spleen)
n.c.
gp33
F
Control
Control+αNK1.1
Grn-/-
Grn-/-+ αNK1.1
LCMV (log10pfu/Spleen)
LCMV (log10pfu/Liver)
LCMV (log10pfu/Lung)
LCMV (log10pfu/Kidney)
LCMV (log10pfu/SC)
LCMV (log10pfu/Brain)
G
DAPI/LCMV-NP
Control
Control+αNK1.1
Grn-/-
Grn-/-+αNK1.1
LCMV-NP (MFI, arbitary units)
H
Control
Control+αNK1.1
Grn-/-
Grn-/-+ αNK1.1
AST (IU/L)
ALT (IU/L)
I
DAPI/α-SMA
Control
Control+αNK1.1
Grn-/-
Grn-/-+αNK1.1
α-SMA (MFI, arbitary units)

Figure 14. NK cell depletion restores defective T cell immunity in Grn$^{-/-}$ mice.

(A) *WT and Grn$^{-/-}$ mice were infected with LCMV-WE. Splenocytes were mixed with RMA/s cells for 16 hours. IFN-γ^{+} NK cells were measured by flow cytometry (n=8-15);*

(B) *Splenic NK cell mediated killing to RMA/s cells was determined by mixing LCMV infected splenocytes with RMA/s cells at indicated effector-to-target ratio for 18 hours (n=12-14);*

(C) *WT and Grn$^{-/-}$ mice were transferred with 10^6 P14^{+} cells, following LCMV infection. The frequency (left) and absolute cell numbers of apoptotic P14^{+} cells were determined at day 2 after infection (n=5-6);*

(D) *WT and Grn$^{-/-}$ mice were treated with anti-NK1.1 antibody at day -1 followed by LCMV infection. Antiviral CD8^{+} T cells in the blood were measured by flow cytometry at indicated time points (n=5-8, * represents the statistical analysis between WT and Grn$^{-/-}$ groups);*

(E) *Splenic IFN-γ^{+}CD8^{+} T cells were determined by incubation of gp33 peptides for 6 hours (n=8-9), n.c. represents no adding of gp33;*

(F) *Virus titers were measured in indicated organs by plaque assay (n=8-9);*

(G) *Sections of snap-frozen liver organs were analyzed for LCMV-NP levels (n=6-8), right panel indicates quantification of fluorescence intensity. Scale bars: 50μm;*

(H) *Serum AST (left) and ALT (right) activities were determined at day 8 after LCMV infection (n=4-5);*

(I) *Sections of snap-frozen liver organs were analyzed for alpha-SMA levels (n=6-8), right panel indicates quantification of fluorescence intensity. Scale bars: 50μm;*

*Data show mean ± s.e.m *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

5. Discussion

In this study, we showed that PGRN limited NK cell cytotoxicity through suppressing cyclin T1 and CDK9 expression in NK cells. *Grn*$^{-/-}$ mice exhibited increased NK cell-mediated regulation of antiviral T cells during LCMV infection, which was associated with prolonged LCMV infection. In turn, NK cell depletion rescued defective T cell immunity and prevented viral persistence in *Grn*$^{-/-}$ mice during infection with LCMV, suggesting its protective role for antiviral immunity.

PGRN is widely expressed in mammalian cells, including immune cells and non-immune cells (583, 584). It is also highly expressed in several disease conditions, such as cancers (510, 585), and bacterial infections (586, 587). In our study, we found that viral infections also lead to increased levels of serum PGRN in the early stages after infections, suggesting that PGRN may have roles in defence againist viral infection. Furthermore, when we transferred the bone marrow cells isolated from *Grn*$^{-/-}$ mice to the WT irradiated hosts, serum PGRN still showed very high levels after LCMV infection, suggesting non-immune cells might also be a big source of PGRN during viral infections. Consistently, the gastric epithelial cells alone could express PGRN during direct contact to bacteria (549, 563), suggesting epithelial cell-intrinsic pathways are also involved in PGRN production.

Many types of cell surface molecules act as inducers of PGRN in mammalian cells. A series of TLRs had been reported to be the determinant of PGRN production during cecal ligation puncture (CLP)-induced sepsis, whereas IFNAR deficient mice displayed comparable levels of PGRN to control mice (587). In our study, PGRN levels were blocked in *Ifnar1*$^{-/-}$ mice during viral infection, suggesting IFN-I might be the determinant of PGRN induction in viral infection models. Tissue-resident macrophages were the main immune cell source of PGRN during cancer and

bacterial infections (552, 588). Consistently, depletion of macrophages with clodronate eliminated PGRN production during LCMV infection, whereas the deletion of dendritic cells or $CD169^+$ cells revealed comparable PGRN levels to their controls, suggesting macrophages might be the main source to PGRN in viral infection models.

Many membrane-expressing molecules were identified to be the receptors of PGRN in both immune cells and non-immune cells. Granulins, but not PGRN, act as a soluble cofactor for TLR9 signals by directly interacting with CpG (564). We also observed reduced IFN-I production in *Grn*$^{-/-}$ mice after both LCMV and VSV infection (data not shown here). It may suggest that PGRN or Granulins act as a co-sensor or co-receptor to viral RNA.

Sortilin was also identified to be the surface receptors for PGRN with the observations that sortilin deficiency resulted in reduced PGRN binding in neurons (525). In addition, sortilin had also been reported to modulate the trafficking of IFN-γ and Granzymes in NK cells, with the observations that sortilin deficiency resulted in enhanced NK cell cytotoxic activity (589). These findings prompted us to suspect that sortilin might be the functional receptor of PGRN on NK cells. However, *sortilin*$^{-/-}$ NK cells expansion and Granzyme B expression were still suppressed by PGRN in vitro (data not shown here), indicating that sortilin may not be the only functional PGRN receptor on NK cells.

Furthermore, EphA2 has also been reported to be another receptor of PGRN (532). Interestingly, EphA2 had also been reported to modulate the NK cell phenotype in patients, with the observations that treatments of solid tumor patients with EphA2 antibodies resulted in inducing activated NK cells in a dose-dependent manner (590). These studies may highly suggest that EphA2 may act as the functional receptor of PGRN on NK cells.

Furthermore, PGRN can competently bind to TNFRs and therefore resolves TNF signals to protect arthritis (526). However, two other papers showed that PGRN didn't inhibit TNF signalling through TNF receptors (527, 528), suggesting that PGRN may indirectly interact with TNFRs. In this study, TNFR deficient NK cells still can be suppressed by PGRN with the lower Granzyme B levels in our case, suggesting that TNFRs may not be the direct receptors of PGRN on NK cells. The functional receptors of PGRN on NK cells remain unclear in this study.

As discussed above, PGRN is highly expressed in many types of cancers. Numerous biological processes for tumor development were affected by PGRN, including promoting cancer cell growth (524), invasion (591), and avoiding cell lysis from NK cells (542, 592). Consistently, incubation with PGRN in cell cultures resulted in suppressed NK expansion by impairing the NK cell cycles. After the binding or uptake of PGRN to cells, the intracellular signals were activated. PGRN biological responses trigger phosphorylation of Akt, Erk1/2, and MAPK pathways (593, 594). However, the activation of Akt or MAPK signalling pathways result into enhanced NK cell activity (595), which is opposite to our phenotype that NK cells were suppressed by PGRN treatment, suggesting the Akt and MAPK signalling pathway may not be involved in PGRN induced NK cell suppression. Once PGRN was secreted into the extracellular space, the PGRN can either be cleavaged into granulins by extracellular proteases (561, 596) or taken up into cells via co-factors or transmembrane receptors (597). Intracellular PGRN or granulins had been reported to interact with the Cyclin T1 (567, 568). CDK9 and Cyclin T1 are the main components of the global transcription elongation factor which termed as the positive transcription elongation factor b (P-TEFb) (598). It will phosphorylate the C terminal domain of RNA polymerase II (RNAPII) and promote the effective elongation of target gene transcription (599, 600). In this study, both Cyclin T1 and CDK9 protein levels were reduced in NK cells following PGRN

incubation, suggesting PGRN had a suppressive role to P-TEFb levels in NK cells. Consequently, the phosphorylation of RNA polymerase II was repressed in NK cells during PGRN treatments, which results in reduced Granzyme B and IFN-γ expression. Consistently, Cyclin T1 and CDK9 promote $CD8^+$ T cell function, since knockdown of Cyclin T1 or CDK9 suppresses T cell Granzyme B and Perforin expression (601). It is known that enhanced IL-2R signalling, leading to induced perforin expression by T cells, is correlated with dramatic recruitment of RNA polymerase II (576), which may give an explanation to the question why just cytotoxic molecules were suppressed but the cell surface receptors showed comparable levels to the PGRN untreated group during IL-2-mediated NK cell expansion in this study.

Since its initial discovery, PGRN has been implicated to be a pro-inflammatory secreted molecule in a variety of diseases including autoimmunity (602), inflammation and infectious diseases (553). Serum PGRN levels were always increased in these disease conditions, whereas PGRN deficient mice exhibited a more severe disease phenotype than WT controls (602). Consistently, PGRN deficiency leads to LCMV virus persistance by reducing the antiviral T cell immunity. These results prompted us to ask whether PGRN has a direct impact on antiviral T cells. It is known that PGRN promotes differentiation of regulatory T cells (526, 578) and Th17 cells (603). In this study, we notified both $CD4^+$ and $CD8^+$ T cells showed comparable levels in *Grn*$^{-/-}$ mice to their controls in all peripheral lymphoid organs, suggesting that PGRN may not directly influence $CD8^+$ T cell development. In addition, enhanced NK cell activity was also observed by the results that *Grn*$^{-/-}$ NK cells displayed higher cytotoxic activity and higher IFN-γ levels to RMA/s cells than NK cells from WT mice after LCMV infection. NK cells act as an important regulatory cell type to adaptive immunity by directly regulating T cells during viral infections (499, 507). These previous findings prompted us to suspect that the reduced antiviral T cell immunity

was mediated by NK cell in *Grn*$^{-/-}$ *mice.* As expected, the reduced antiviral T cell responses were rescued by NK cells depletion in *Grn*$^{-/-}$ mice, leading to faster pathogen elimination. Type I interferons could induce a variety of inhibitory NK cell ligands and also MHC-I molecules on antiviral T cells to protect them against cytotoxicity (364, 365). In this study, we didn't observe any MHC-I difference on antiviral CD8^{+} T cells between WT and *Grn*$^{-/-}$ mice after LCMV infection, suggesting PGRN may not modulate T cell MHC-I expression to avoid NK cell cytotoxicity.

In summary, we demonstrated that macrophage-derived PGRN played a suppressive role to NK cell-mediated antiviral T cell lysis.

6. References

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