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NK cells negatively regulate CD8$^+$ T cells during chronic viral infection in FcεRIγ-dependent manner

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1 INTRODUCTION

1.1 The immune system

Germs penetration, causing disease and pathogens eradiation are outcome of interplay between pathogenicity of the organism (the virulence factors) and the integrity of host defence mechanisms. The immune system encompasses an interactive specialized network of cells and molecules spread throughout the body, which are constantly on the lookout for invaders to spot and mount them. Upon host penetration, pathogens encounter three levels of immunity: anatomical and physiological barriers, innate immunity, and adaptive immunity. Anatomical and physiological barriers are the key first line of defense against pathogens. These barriers include intact skin, vigorous mucociliary clearance mechanisms, low stomach pH and bacteriolytic lysozyme in tears, saliva and other secretions. The innate or natural response occurs non-specifically every pathogen encounter in a timely manner. Innate immunity, which depends on a limited repertoire of invariant receptors, magnify the protection served by anatomical and physiological barriers. The second arm of immune system is the acquired or adaptive response that develops slowly lasting several days and weeks. The adaptive immune response has a memory, which is a cardinal feature, and enable the cellular components to respond vigorously to subsequent exposure of infection (Delves and Roitt, 2000, Turvey and Broide, 2010, Parkin and Cohen, 2001).

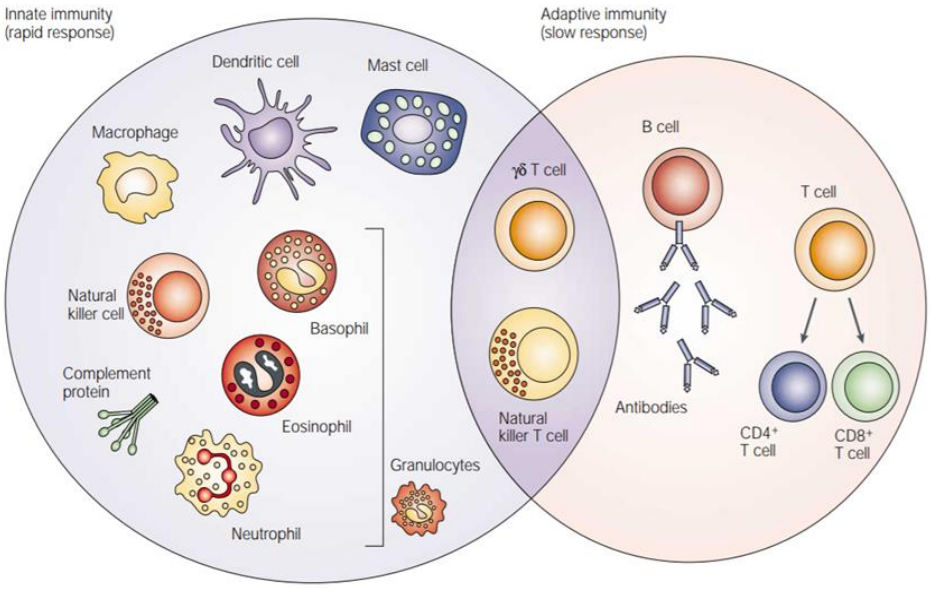

Fig 1.1: Arms of the immune system
Dranoff G (2004): Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer

1.1.1 The structure of the immune system

Bone marrow and thymus comprise the primary or central lymphoid organ and are mainly responsible for development of all immune cells derived from hematopoietic stem cells, process known as Haematopoiesis. Bone marrow serves as a reservoir for the production of hematopoietic stem cells and is the primary site for B cells development in mammals (Ruddle and Akirav, 2009). Whereas the thymus provides the main venue for development and education of T cell progenitors. Secondary lymphoid organs include spleen, lymph nodes and tonsils and function as microenvironment for effective immune response regulation and hemostasis (Pabst, 2007). The spleen is considered as a primary lymphoid organ since the late stage of B cell development occurs there, nevertheless is basically secondary lymphoid organ (Boehm and Bleul, 2007). Tertiary lymphoid tissues or so called ectopic lymphoid follicles are aggregations of lymphocytes and stromal cells in an organized structure outside of secondary lymphoid organs and found in inflamed sites in autoimmune diseases (Shipman *et al.*, 2017).

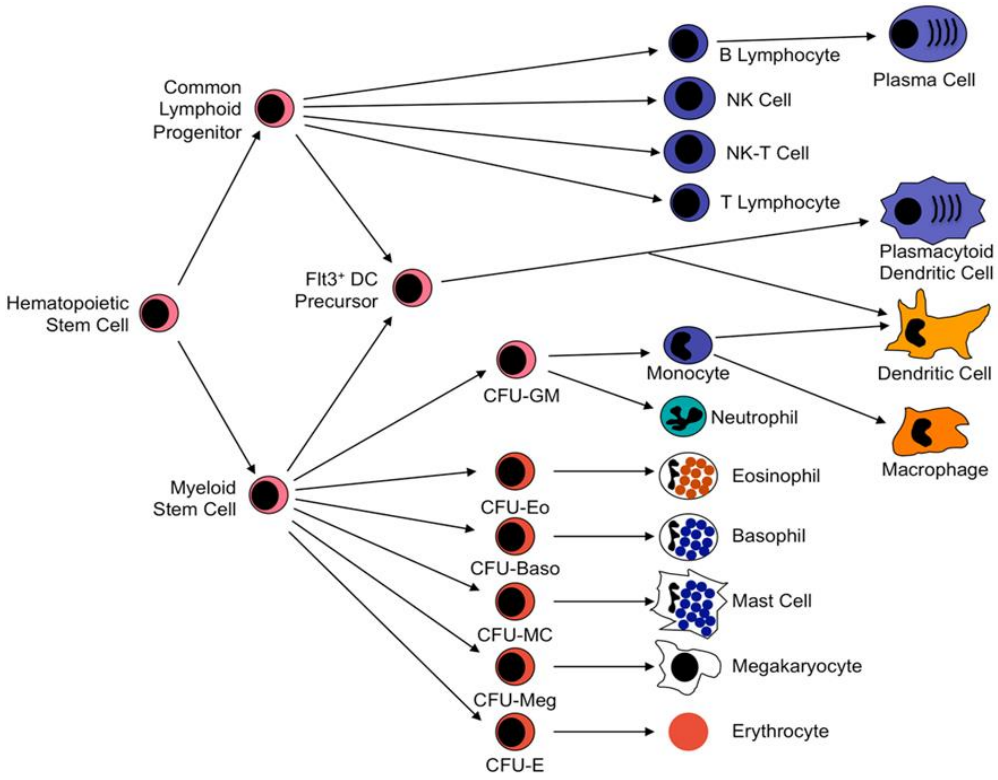

Fig 1.2: Haematopoiesis
Chaplin DD (2010): Overview of the immune response. J Allergy Clin Immunol

1.1.2 Innate immune response

The central components of innate immune system are considered evolutionarily conserved among vertebrates (Buchmann, 2014). Innate arm of immune defense in mammals encompasses specialized phagocytic cells (neutrophils, monocytes, and macrophages), cells that release inflammatory mediators (basophils, mast cells, and eosinophils), and natural killer cells (Galli *et al.*, 2011). The innate immune system detects the invaders through germline-encoded receptors (e.g. pattern recognition receptors [PRRs] such as toll-like receptors [TLRs]). Innate immune responses can be driven through cell-dependent mechanisms (e.g. phagocytosis and cytotoxicity) or via molecular components or secreted factors such as; acute phase proteins, complement factors and cytokines/chemokines (Gasteiger *et al.*, 2017).

Pluripotent hematopoietic stem cells differentiate in bone marrow into common lymphoid or common myeloid progenitor cells. B cell, T cell, and NK cell lineages originate from lymphoid stem cells, whereas neutrophils, monocytes, eosinophils, basophils, mast cells, megakaryocytes, and erythrocytes stem from myeloid progenitor cells. Monocytes give rise further into macrophages in peripheral tissues. The common precursor to macrophages, monocytes and DCs is the macrophage-DC progenitor (MDP). Classical or plasmacytoid dendritic cells develop primarily from a DC precursor derived from lymphoid or myeloid stem cells and characterized by its expression of the Flt3 receptor (Chaplin, 2010, Kushwah and Hu, 2011).

1.1.2.1 Type I Interferons

Interferons (IFNs) are secreted polypeptides that are released by infected cells, macrophages, DCs, fibroblasts, epithelial cells. Conventionally, three major IFN families exist; The type I,II, and III interferons (Randall and Goodbourn, 2008). Type I IFNs mainly include 13-14 subtypes of IFNα in human and murine models and are produced mainly from haematopoietic cells especially pDCs, which is a potent producer, and IFNβ that is secreted by virtually all host cells (Pestka *et al.*, 2004). Recognition of microbial stimuli like pathogen-associated molecular pattern (PAMPs) by PRRs (e.g. TLR) elicit the expression of IFN-stimulated genes (ISGs) to produce IFN I via multiple signalling pathways. Once Type I IFNs are produced and engaged to their receptors, several downstream signalling pathways are induced to result in pathogens dissemination restrain and other biological effects (Ivashkiv and Donlin, 2014, McNab *et al.*, 2015).

The type II IFN family comprises IFNγ that is mostly produced by T cells and natural killer (NK) cells. The type III IFN family ,which encompasses IL-29, IL-28A and IL-28B, have comparable functions to cytokines of the type I IFN, and are secreted by virtually all nucleated cells and they resemble IL-10 in term of structure but their targets (receptors) are restricted to epithelial cells and hepatocytes (Witte *et al.*, 2010). Type I IFNs have a diverse functions ranging from elicitation of autonomous antimicrobial states of infected cells to amplification of the innate and adaptive immune response (McNab *et al.*, 2015). Aside to their cardinal role in limiting the viral infection, Type I IFNs have a substantial role in shaping the bacterial infection, cancer and autoimmune diseases in protective or detrimental manner (Trinchieri, 2010).

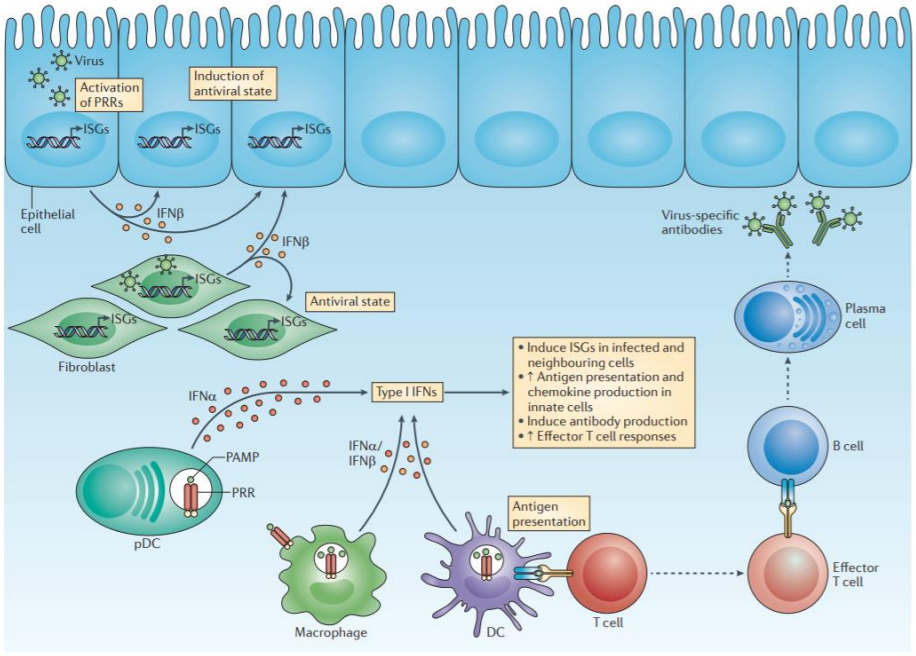

Figure 1.3: Type I interferon responses
Ivashkiv LB *et al.*, (2014): Regulation of type I interferon responses. Nat Rev Immunol

1.1.2.2 Type II Interferon

Type II Interferon (IFN-γ) is widely used in vitro setting to assess the functionality of virus-specific T cells upon acute and chronic infections. IFN-γ is produced mainly by T cells along with other immune cells such as $CD4^+$ T cells, B cells NK cells. It has a plethora of

paradoxical functions in terms of both the pathogen eradication and orchestrating the innate and adaptive immunity (Schroder *et al.*, 2004, Demers *et al.*, 2013). Of these, it exert a robust antiviral effect by enhancement the antigenic nucleoprotein presentation and recognition by $CD8^+$ T cells (Groettrup *et al.*, 1996), upregulating the expression and stability of MHC class I molecules directly, or indirectly by the TAP transporter proteins upregulation (Demers *et al.*, 2013, Epperson *et al.*, 1992). Furthermore, IFNγ increase the expression of TNF-α receptors and Fas/FasL resulting in apoptosis of virally infected cells (Tsujimoto *et al.*, 1986, Xu *et al.*, 1998). In the context of innate and adaptive immunity, IFNγ secreted from NK cells induce the transcription factors, which stimulate the differentiation of naïve $CD4^+$ T cells to the Th1 subset, and this stimulation is amplified by the action of IL-12 from macrophages and DCs. Moreover, IFNγ released from Th1 cells augments this response and inhibits the development of Th2 cells. In other context, T cells activate the macrophages as well as antibody secretion and isotype switching (Abbas *et al.*, 2018). Conversely, IFN-γ is negatively regulated by the action of IL-4, IL-10, transforming growth factor beta, and glucocorticoids (Schroder *et al.*, 2004)

1.1.2.3 Natural Killer (NK) cells

Natural killer cells are a subset of bone marrow-derived large granular lymphocytes and act as innate immune system sentinels by virtue of their ability to release cytokines and to mediate cytolytic activity against tumor cells as well as virus-infected cells (Vivier *et al.*, 2008). NK cells were first described in the early 1970s by their ability to lyse tumor cells without prior stimulation (Herberman *et al.*, 1975, Kiessling *et al.*, 1975). Along with innate lymphoid 1 cells, NK cells are prototypical member of the innate lymphoid cells (ILC) family constituting group 1 ILCs, and they are considered the innate counterpart for $CD8^+$ T cells (Spits *et al.*, 2016). In mice, NK cells are widely distributed in lymphoid and non-lymphoid organs. The largest number of murine NK cells presents in spleen, whereas the highest frequency of NK cells inhabit the lung (Gregoire *et al.*, 2007a). In human, it was found that lymph node NK cells outpace blood NK cells by 10:1, in contrary to 1:1 ratio in mouse, possibly due to the expression of CCR7 on a subset of human NK cells (Ferlazzo *et al.*, 2004). Adaptive immunity represented by T and B cells express recombination-activating genes (RAGs) that mediate the rearrangement of genes encoding antigen recognition receptors.

After antigen exposure and priming, antigen-specific T and B cells undergo dramatic expansion, which is described as effector phase. Upon antigen clearance, most of these effector cells undergo apoptosis during the contraction phase, but 5-10% survive and progressively differentiate into long-lasting memory cells that mediate a faster and more robust antigen-specific response than naïve cells once reinfection (Chang *et al.*, 2014, Williams and Bevan, 2007). Unlike the adaptive arm of immune system, innate immunity respond rapidly against pathogens and transformed cells prior sensitization and considered short-lived receptors (Liu and Cao, 2016). Innate immune cells including ILCs and NK cells do not express antigen receptors or undergo clonal selection and expansion when stimulated, but rather express various germ-line encoded activating and inhibitory receptors (Biron *et al.*, 1999, Peng and Tian, 2017). Recently, an emerging evidence of studies showed that NK cell-mediated immune responses share functional features with adaptive immunity, and NK cells acquire immunological memory similar to T and B cells. Three distinct types of memory were described; hapten-specific NK cell memory, virus-specific NK cell memory and cytokine-induced NK cell memory (O'Sullivan *et al.*, 2015, Cerwenka and Lanier, 2016).

NK cells react to prompt signals from insult like infected, stressed or neoplastic cells without prior sensitization and produce an array of cytokines, which direct the developing immune response adaptively to the peril nature (Biron *et al.*, 1999, Gregoire *et al.*, 2007b). A balance drives this regulatory capability of NK cells between signals that are generated from activating and inhibitory receptors which are stochastically expressed on NK cells, culminating in augmented or restrained effector functions (Lanier, 1998). Thus, the capability of NK cells may be controlled or unleashed to rheostat or magnify immune responses in disease prevention and therapy.

1.1.2.3.1 NK cells effector functions

The quality and quantity of NK cell cytotoxic and cytokine responses depend on the cytokine microenvironment, as well as on conjugation with T cells, dendritic cells (DCs) and macrophages. Type I IFN, IL-2, IL-12, IL-18 and IL-15 are potent mediators of NK cell effector function (Vivier *et al.*, 2008). NK cells kill the target cells mainly by cytotoxicity beside cytokines release. More in depth, those innate sentinels adopt multiple cytotoxic means to mount the peril. Firstly, exocytosis of perforin/granzyme-containing granules (perforin and granzyme–mediated mechanism) which is the key mean to get rid

of infected and cancerous cells. Once the balance between activating and inhibiting receptors skewed toward activation, perforin facilitates the delivery of granzyme B into the cytosol of the target cell, which is serine proteases cleave several intracellular substrates such as procaspases to initiate the classical apoptotic pathways (Smyth *et al.*, 2002, Yoon *et al.*, 2015). Secondly, the Death-receptor-mediated apoptosis CD95 (FAS) - CD178 (FAS ligand) pathway in which three main bounded or secreted cytokines are involved; FAS ligand (FASL)(CD178), tumor necrosis factor (TNF) and TNF-related apoptosis inducing ligand (TRAIL) which they spark signalling unit and the subsequent activation of caspases that promote the apoptotic process of the (Fas) CD95-expressing target cell (Smyth *et al.*, 2002, Yoon *et al.*, 2015). Thirdly, the antibody-dependent cellular cytotoxicity (ADCC) mechanism, in which CD16 (FcγRIIIA) is expressed on NK cells along with other leukocytes and is considered receptor for Fc portion of IgG antibodies. Upon binding of human IgG to antigen - bearing cells, IgG antibody is identified by CD16 and elicit NK cell for killing of antibody-coated target cells. Antibody-dependent activation of NK cells is one of the mechanisms against viral infections such as; influenza virus, herpes simplex virus type 1 and HCMV (Hammer *et al.*, 2018, Yoon *et al.*, 2015, Morvan and Lanier, 2016).

Other than cytotoxicity functions, NK cells have a prominent role in cytokine production which participates in the regulation of myeloid hematopoiesis and activation of myeloid**,** and monocytes via production of granulocyte macrophage–colony-stimulating factor (GM-CSF), IL-3, IL-5,IL-10, IL-13, IFN-γ and tumor necrosis factor alpha (TNF-α) (Loza *et al.*, 2002, Smyth *et al.*, 2002). IFN-γ has a principal role in antiviral defense especially against murine cytomegalovirus (MCMV) and influenza virus. IFN gamma production is a consequence of IL-12, IL-15, IL-1 as well as TNF. Moreover, nitric oxide, which is released from macrophages in response to interferon gamma, is reported to regulate NK cells regulatory functions as well as its dramatic anti-microbial properties. Furthermore, TNF confers antiviral effect to lesser extent as it is released from a wide range of immune cells (Biron *et al.*, 1999, Smyth *et al.*, 2002). Additionally, NK cells can make certain chemokines (low molecular weight cytokines) which has chemoattractant, pro-inflammatory functions and antiviral effects in case of HIV infections (Biron *et al.*, 1999).

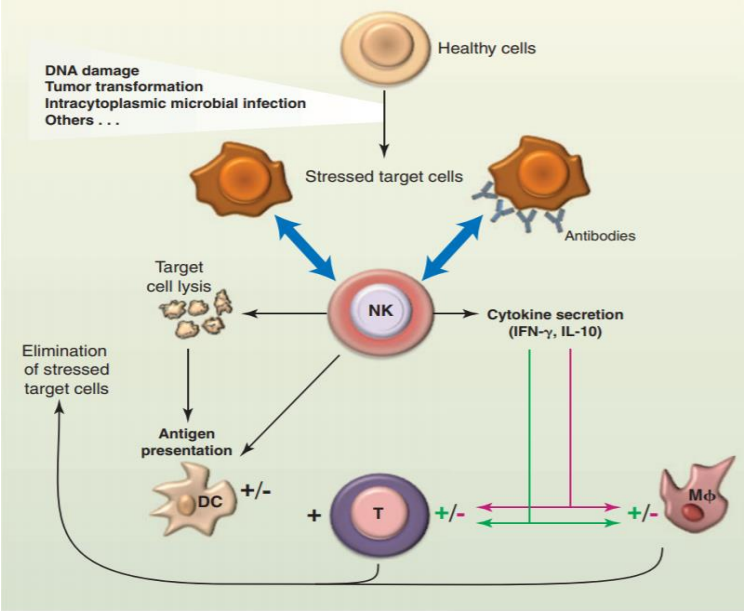

Figure 1.4: Overview for NK cells biological functions
Vivier E *et al.*, (2011): Innate or adaptive immunity? The example of natural killer cells. Science

1.1.2.3.2 NK cells and viruses

NK cells have a substantial role in controlling viral infections. Examples of this abound, arenaviruses (LCMV), the herpesviruses, (HSV) and (MCMV), the orthomyxoviruses (influenza virus), and the picornaviruses (Coxsackie virus) (Biron *et al.*, 1999). Two major prototypical viruses were investigated in the murine models in terms of NK cells- mediated recognition; LCMV and MCMV. In the context of MCMV infection, which is described as NK cells sensitive, the viral protein m157 is exquisitely recognized by the activating receptor Ly49H that triggers the population expansion of $Ly49H^{+}$ NK cells (Hammer *et al.*, 2018). DNAM-1, which is a costimulatory receptor, is upregulated during infection and work synergistically with Ly49H and NKG2D. By the same token, DNAM-1 and NKG2D do not sense the virus directly unlike the KIRs and NKG2C in humans, as well as activating receptors of the Ly49 family in mice and NK1.1 in both models (Nabekura *et al.*, 2017, Nabekura *et al.*, 2014). In the context of immunoevasion, MCMV also encodes proteins namely; m152, m04, m06 that inhibit the expression of MHC I and hence restoring the 'missing self' phenomenon and allowing the engagement of inhibitory receptors Ly49 (Babic *et al.*, 2010). Further, MCMV encodes proteins that downmodulate expression of the ligands for NKG2D and thereby compromising the NK cells (Arapovic

et al., 2009). Summarizing the privileges of NK cells during MCMV infections, the former has a key role in the control of HCMV and MCMV and is characterized by induced cytotoxicity and interferon gamma production (Biron *et al.*, 1999), as noticed by the enhanced susceptibility to HCMV and impaired control to MCMV in hosts lacking NK cells (Biron *et al.*, 1989, Bukowski *et al.*, 1984).

In striking contrast, NK cells has modest role in controlling the LCMV infections mainly by cytotoxicity and slightly by IFN gamma in the periphery (Biron *et al.*, 1999), and hence LCMV is best described as NK cells resistant, as it is not directly calibrated by NK cells. Nevertheless, depletion of NK cells can profoundly alter the patterns of LCMV pathogenesis and persistence, not due to direct effect of NK cells on LCMV, but rather, by virtue of ability of the NK cells to modulate the T cell response (Welsh and Waggoner, 2013). In the LCMV model, depletion of NK cells thwarts the severe pathology in the medium dose of LCMV and results in viral resolving, indicating that NK cells are detrimental to T cells in this dose. In other direction, NK cells ablation in high viral dose model culminate in fulminant outcome, which means the important role of NK cells to maintain the viral persistence. Shortly, the NK cells are rheostat-like regulators of the magnitude and functionality of T cells responses that determine the fate of viral load (Welsh and Waggoner, 2013).

1.1.2.3.3 Activating and inhibitory receptors of natural killer cells

Activation of NK cells occurs through delicate interaction between myriad cytokines, such as, type I interferons (IFNs), IL-2, IL-12, IL-15, and IL-18 (Crouse *et al.*, 2015), or by direct triggering of toll-like receptors (TLRs) or *via* responding to activating and inhibitory NK cell receptor ligands upon cellular changes or infections (Pallmer and Oxenius, 2016). NK cells have to be educated by detection of host MHC class I molecules by their cognate inhibitory receptors in a process called licensing or tuning in which they acquire the capacity to identify target cells with low MHC class I expression (‘missing self’ recognition). In this process, NK cells circumvent the recognition of their cognate cellular ligands and consequent autoimmunity (Vivier *et al.*, 2011, Raulet and Vance, 2006). NK cells also adapt to their environment through the process of ‘priming’ by cytokines, such as IL-15 (trans-presented by DCs), IL-18 and IL-12 as well as type I IFN (Vivier *et al.*, 2008). Conventionally, downregulation of ligands for NK inhibitory receptors (such as MHC-I

molecules, known as 'missing self'), or an upregulation of ligands for activating NK receptors is the major determinants of NK cells signal upon infections and cellular changes (Crouse *et al.*, 2015). On one hand, NK cells avoid healthy cells that express self-MHC class I molecules and low amounts of stress-induced self-molecules causing protection or tolerance. On the other hand, NK cells selectively kill distressed target cells that lose or down-regulate MHC class I molecules in a process called missing-self recognition. Moreover, they can cull the target cells due to upregulation of stress-induced self-molecules or stimulatory ligands that overcome the constitutive expression of inhibitory receptors and is termed induced-self recognition (Raulet and Vance, 2006).

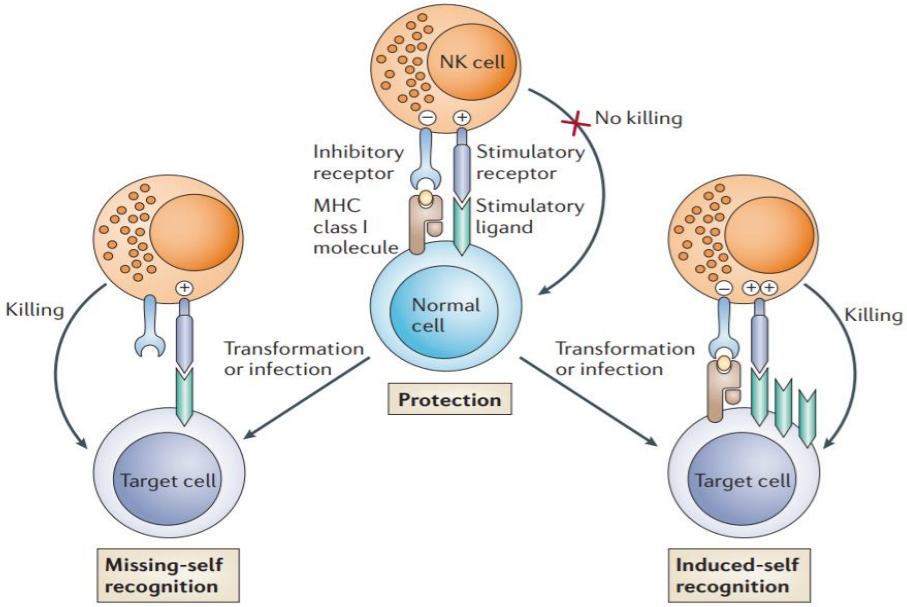

Figure 1.5: The dynamic equity between stimulatory and inhibitory signals in NK cells
Raulet DH *et al.*, (2006): Self-tolerance of natural killer cells. Nat Rev Immunol

1.1.2.3.4 Natural cytotoxicity receptor 1 (NCR1)

A milieu of inhibitory, costimulatory, and activating receptors orchestrate the killing function of natural killer cells. Among the activating NK cell receptors are NKp46, a unique member of the natural cytotoxicity receptors (NCRs) family, which also includes NKp30 and NKp44. Moreover, NKp46 has the only mouse ortholog, named Ncr1, and is the main activating receptor expressed in both resting and activated NK cells similar to NKp30 and unlike the NKp44 that is expressed only on activated NK cells (Moretta *et al.*, 2000, Biassoni *et al.*, 1999). NKp46/Ncr1 directly recognizes the hemagglutinin (HA) proteins of influenza viruses (Mandelboim *et al.*, 2001), and other viruses like poxviruses, and Newcastle disease

viruses (Jarahian *et al.*, 2009). Recently, some non-viral ligands have been elucidated such as; complement factor P as well as surface protein on healthy pancreatic β cells. Albeit the identities of the cellular ligands of NKp46/Ncr1 are still elusive, it is putative that NKp46/Ncr1 is involved in an array of immunological activities (Narni-Mancinelli *et al.*, 2017, Gur *et al.*, 2010). While NKp46 expression is specific for NK cells, NKp30 and NKp44 can also be found on subsets of T cells (Watzl and Long, 2010).

In contrary to T cell receptors (TCRs) and immunoglobulins, NKp46 does not undergo any somatic recombination in order to be effective (Hudspeth *et al.*, 2013). NKp46 is type I transmembrane ™ receptors and consists of four main domains which are a signal peptide, two constant type 2 (C2-type Ig) domains in extracellular region which bind to viral glycoprotein (unlike the NKp30 and NKp44 that contain one domain), a transmembrane region expressed as disulfide-bonded heterodimers of FcRγ and CD3ζ that bind to NCR1 through the formation of a membrane-embedded salt bridge, and an intracellular tail which does not contain any immunoreceptor tyrosine-based activation motifs (ITAM) (Hollyoake *et al.*, 2005).

An earlier study suggests that, NKp46 associates with the ITAM-bearing transduction polypeptides (FcRγ and CD3ζ), and is thought to be stabilized by Arginine, a positively charged amino acid present in the transmembrane domain of NKp46 which is presumably involved in, and facilitates the association and stabilization of the NCR with signalling partner chains containing a negative charge in their transmembrane region (Pessino *et al.*, 1998, Vitale *et al.*, 1998, Walzer *et al.*, 2007, Hollyoake *et al.*, 2005).

Moreover, human CD16, NKp30 and NKp46 can associate with homodimers or heterodimers of the FcRγ and CD3ζ adapter proteins. In contrast, mouse NKR–P1C and mouse CD16 associate with homodimers of mouse FcRγ, but not CD3ζ. Unlike other receptors that associate with FcRγ and CD3ζ, CD16 has an acidic residue, instead of a basic amino acid in its transmembrane (Lanier, 2003).

The gene coding for NKp46 (NCR1) is mapped on human chromosome 19 in a region called Leukocyte Receptor Complex (LRC), telomeric to the KIR multigene family (Biassoni *et al.*, 2003) while the murine counterpart is located on chromosome 7(Biassoni *et al.*, 1999), whereas NKp30 (NCR2) and NKp44 (NCR3) genes are located on chromosome 6 in human (Hudspeth *et al.*, 2013).

Upon ligation of NCR activating receptors with their desired ligands, they transduce signals through the association with adaptor molecules, such as CD3ζ, FcεRIγ and DAP-12 which contain in their intracytoplasmic region ITAM that upon phosphorylation transduce activation via $p72^{syk}$ and ZAP70 cytoplasmic PTK. Contrarily, other receptors such as NKG2D use the associated DAP-10 polypeptide to trigger via the PI-3 kinase pathway (Lanier, 2003, Biassoni *et al.*, 2003).

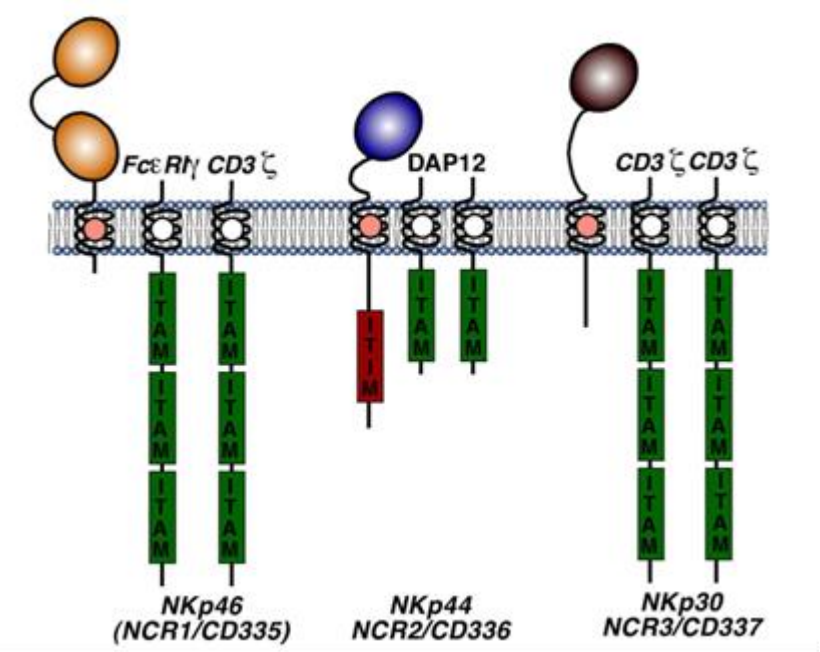

Figure 1.6: Natural cytotoxicity receptors
Hudspeth K *et al.,* (2013): Natural cytotoxicity receptors: broader expression patterns and functions in innate and adaptive immune cells. Front Immunol (modified).

1.1.3 Adaptive immune system

Cellular and humoral constituents drive the adaptive immune response. Acquired responses involve the proliferation of antigen-specific B and T cells, which occurs when the surface receptors of these cells bind to antigen. Specialized cells, called antigen-presenting cells, display the antigen to lymphocytes and collaborate with them in the response to the antigen. B cells secrete immunoglobulins, the antigen-specific antibodies responsible for eliminating extracellular microorganisms. T-cells help B cells to make antibody and can eradicate intracellular pathogens by activating macrophages and by killing virally infected cells. Innate and acquired responses usually collaborate to get rid of pathogens. More in depth, the cellular adaptive immunity can be subcategorized into; Type I, II and III immunity. Type I

immunity is orchestrated by (ILC1 and NK cells), $CD8^+$ T cells, and T_H1 cells, which mediate immune response against intracellular microbes *via* intense phagocytes activation and through secretion of interleukin (IL)-2, interferon-γ, and lymphotoxin-α. Contrarily, Type II immunity are rich in mast cell, basophil, and eosinophil and driven by ILC2s, T_C2 cells, and T_H2 cells by secretion of IL-4, IL-5, and IL-13, resulting in antibody production against helminthes and other non-microbial stimuli. Type III immunity consists of ILC3, cytotoxic and helper T cells producing IL-17, and IL-22, which activate phagocytes, recruit neutrophils and generate epithelial antimicrobial responses, to mount the extracellular bacteria and fungi Moreover, type I and III immunity mediate autoimmune diseases, whereas type II responses can elicit allergic diseases (Spellberg and Edwards, 2001, Annunziato *et al.*, 2015) .

1.1.3.1 T cells

Conventionally, mature T lymphocytes express either CD4 or CD8. $CD4^+$ T cells are called T helper and $CD8^+$ T cells are termed cytotoxic T cells. Antigen-specific $CD4^+$ and $CD8^+$ T cells exist at very low frequencies at resting state. After activation by infection or immunization, naive T cells undergo clonal expansion, resulting in a higher population of antigen-specific cells with effector function. In comparison between $CD8^+$ and $CD4^+$ T cells, the later has a lower rate for division and require more time for antigen exposure to set off the proliferation. Antigen recognition by T cells induce cytokine secretion especially IL-2, clonal expansion and differentiation of T cells into effector or memory cells. During the effector phase of immune response, the effector $CD4^+$ T cells produce cytokines with several actions, such as recruitment and activation of B–cells and macrophages, whilst $CD8^+$ T cells respond by killing and secreting inflammatory cytokines (Abbas *et al.*, 2018, Owen and Kuby, 2013, De Boer *et al.*, 2003).

Both $CD8^+$ and $CD4^+$ T cells response to acute or chronic infection can generally be broken down into three consecutive phases. First, the induction and effector phase, in which naive T cells precursors are primed, undergo dramatic expansion, acquire effector function, traffic to sites of infection, and mediate pathogen clearance. Second, the contraction phase, in which (90-95 %) most effector CTL die in a process called activation-induced cell death (AICD) and occur after antigen clearance, leaving behind 5%–10% as long-lived memory cells that are polyfunctional in absence of antigen in case of acute infection. Dislike, in chronic persistent infection, T cell responses persist for a long time and are subject to

negative regulation, lose polyfunctionality in a hierarchical manner, and become antigen dependent rather than developing the ability to persist long-term via antigen-independent self-renewal. Third, the memory maintenance phase is characterized by a stable pool of memory cells lasting for years which could be followed by speedy recall in case of pathogen re-encounter (Ahmed and Gray, 1996, Virgin *et al.*, 2009, Williams and Bevan, 2007).

1.1.3.1.1 T cell activation

In the course of alloimmune responses, both naïve and memory T-cells interact with dendritic cells (DCs) to form the immune synapse, which spark the T lymphocyte activation over three signals (Bromley *et al.*, 2001). The first involves T-cell receptor triggering by antigen presentation in the context of MHC molecules on the surface of DCs or other APCs. The second signal ,which involves the stabilization of synapse and is not antigen-specific, is mediated through adhesion molecules and the generation of signals *via* costimulatory molecules present on the surface of APCs and T cells, B7/CD28 and CD40/CD40L pathways are the best described in T-cell activation. CD80/CD86 (B7.1/B7.2) on APCs interact with their receptor, CD28, on T cells to generate activating signals, while interaction with cytotoxic T lymphocyte–associated protein 4 (CTLA4) generates inhibitory signals. The costimulatory molecule in turn activates the transduction pathways such as; the Nuclear Factor-κB pathway culminating in production of numerous molecules, including interleukin-2 (IL-2), CD25 (IL-2 receptor) and CD40 Ligand (Snanoudj *et al.*, 2007). Signal 3 is defined by the secretion of cytokines by APCs, which signal via cytokine receptors on T cells in order to proliferate and polarize them toward an effector phenotype. Regarding the CD40/CD40L pathway, which is a member of TNF family, CD40 is expressed on all APC, and CD40L on activated $CD4^+$ T-cells, subset of $CD8^+$ T cells and NK cells. Stimulation of CD40 triggers antibody production by B cells and strongly induces B7 and MHC expression on APCs (van Kooten and Banchereau, 1997).

The signal strength that T cells receive by engagement with APCs is determined by three factors: the concentration of peptide–MHC complexes and co-stimulatory molecules which determine the rate of T-cell receptor and the extent of signal amplification, respectively, and the duration of the T-APCs engagement, which determines for how long signals accumulate (Lanzavecchia and Sallusto, 2002).

In the context of the CD4 T cells role in helping $CD8^+$ T cells , an antigen-presenting cell (APC) uptake cellular antigen by phagocytosis of an apoptotic or necrotic cell that carries helper (MHC class-II-restricted) and killer (MHC class-I-restricted) antigens. Phagocytosed antigen is presented to $CD4^+$ T cells, which activate the APC through CD40–CD40-ligand (CD40L) interactions. The activated DC can then promote the $CD8^+$ T cell response, generating cytotoxic effector T cells and memory cells. In contrast, certain infectious agents, viruses or bacteria, might override the need for $CD4^+$ T-cell recognition of antigen on the DC by stimulating Toll-like receptors (TLRs) directly, or by causing the release of inflammatory cytokines such as interleukin-1 or type 1 interferons (Bevan, 2004).

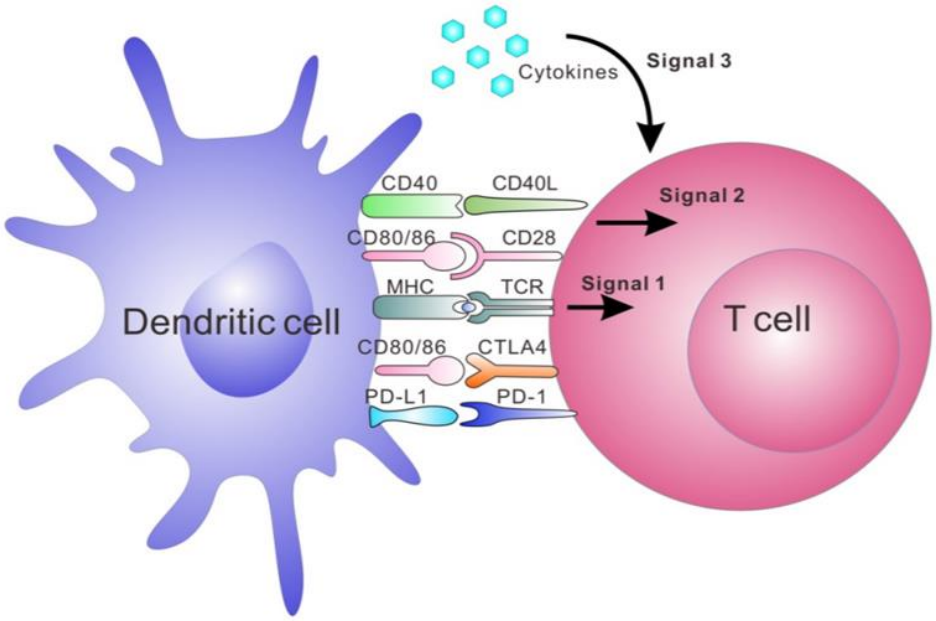

Figure 1.7: A paradigm of T cells and DCs circuit
Wang C *et al.*, (2017): Bioengineering of Artificial Antigen Presenting Cells and Lymphoid Organs. Theranostics.

1.1.3.1.2 Cytotoxic T ($CD8^+$ T) cells

After antigen priming, activated $CD8^+$ T cells receive signals from multiple cytokines such as IL-2, which induces Blimp-1 expression which cooperates with other transcription factors to promote $CD8^+$ (CTL) T cell effector function, migration and exhaustion also IL-12 promotes effector T-cell development via T-bet induction (Zhang and Bevan, 2011). The activated $CD8^+$ T cells mediate a variety of effector functions; cytotoxicity and cytokines production (Williams and Bevan, 2007). Among the cytokines produced are, Interferon-γ, Interleukin-2 as well as TNF-α, which have a broad spectrum of function on the antigen as

well as on immune cells for the sake of invaders eradication (Demers *et al.*, 2013). In term of cytotoxicity as an effector function, CTL adopt two major killing mechanisms; the perforin –mediated which operate perforin to create pores in the target membrane and is co secreted with granzymes that enter the cell and culminating in caspases-3 and -8 activation and hence target cell apoptosis (Cullen and Martin, 2008). The other mechanism is Fas ligand (FasL) –based which involves cross-linking of the cell surface death receptor Fas expressed on target cells induced by cell surface FasL expressed on CTL, leading to activation of caspase-9 which in turn activates caspase-3 (Strasser *et al.*, 2009, Hassin *et al.*, 2011).

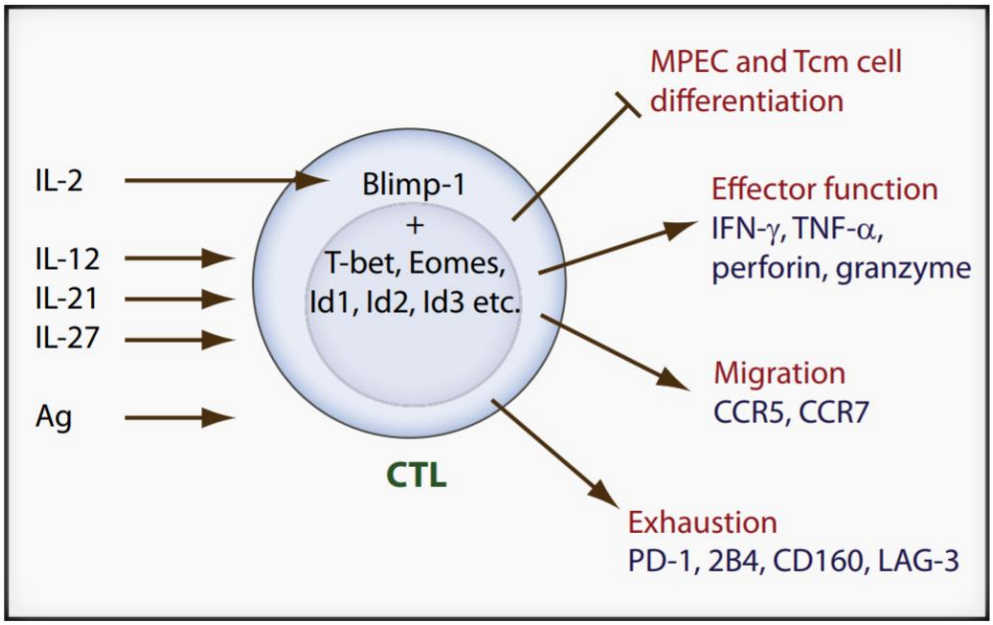

Figure 1.8: Effector CD8+ T cell Differentiation
Zhang N *et al.*, (2011): $CD8^+$ T Cells: Foot Soldiers of the Immune System. Immunity

1.1.4 NK cells crosstalk with immune cells

The activation of NK cells concur with T cells activation early during the course of infection and proximal to the T cells activation sites (Mempel *et al.*, 2004). In turn, NK cells have double-edged sword effect in controlling T cells response directly or indirectly (Crouse *et al.*, 2015). To extend the generality, NK cells can regulate T cells response obliquely by the aid of DCs. For instance, direct NK-DC cells contact which is further amplified by the IFN gamma and TNF release leads to DCs calibration via upregulation of co-stimulatory molecules CD86, CD83, CD80, HLA-DR, and (CCR7) on DCs and augmented the production of IL-12 which in turn enhanced the $CD8^+$ T cells response and tumor regression

in a CD4 independent way. Likewise, NK cell killing of target cells releases antigen for cross presentation and elicitation of T cell responses (Gerosa *et al.*, 2002, Adam *et al.*, 2005, Mocikat *et al.*, 2003, Krebs *et al.*, 2009). NK cell crosstalk with B cells has an impact on T cells response as this engagement can alter the antigen load (Yuan, 2004).

Directly and apart from DCs involvement, IFNγ secreted by migrated NK cells in LN assist in $CD4^+$ differentiation into IFNγ-producing Th1 and their polarization. Also, human-derived NK cells from secondary lymphoid tissue, which secrete higher levels of IFN-γ than blood NK cells, are able to stimulate *in vitro* polarization into the Th1 phenotype (Martin-Fontecha *et al.*, 2004) (Morandi *et al.*, 2006). In another experimental setting, suppression of TGF-β signalling in NK cells leads to NK cell produced IFNγ mediated polarization of Th1 cells, and thus protection against infection (Laouar *et al.*, 2005).

In stark contrast, NK cells can impede the T cell response indirectly in a DC-mediated manner. NK cells may calibrate T cell responses by dampening the availability of antigen-presenting APCs. For example, NK cell ablation in the course of chronic LCMV infection results in enhanced ability of APCs to prime $CD8^+$ T cell responses in primary phase with no effect on the quality or quantity of DCs, suggesting the specific killing of LCMV infected APCs (Cook and Whitmire, 2013). Similarly, NK cells, which are primary target for MCMV infection, can directly recognize and eliminate the m157-bearing infected DCs culminating in reduced $CD4^+$ and $CD8^+$ T cells responses (Andrews *et al.*, 2010). Reciprocally, NK cells secrete IL-10 following MCMV infection culminating in curtailed $CD8^+$ T cell response and hence protection from immunopathology (Lee *et al.*, 2009).

NK cells can also negatively regulate T cell responses directly by their ability to recognize and kill activated T cells as have been shown in numerous emerging studies conducted in humans as well as murine models following viral infections or immunization settings (Crouse *et al.*, 2015). To exemplify, NKG2D activated NK cells engage their putative ligands on T cells resulting in T cells elimination by perforin-mediated killing in human and mouse models (Rabinovich *et al.*, 2003, Lang *et al.*, 2012, Cerboni *et al.*, 2007). Likewise, NK cell depletion during early LCMV infection control viral load by inhibiting specific elimination of activated $CD4^+$ T cells, which subsequently promoted $CD8^+$ T cell responses (Waggoner *et al.*, 2011). Commensurately, depletion of NK cells resulted in increased numbers of responding T cells, including increased amounts of central memory T cells (Soderquest *et al.*, 2011). In line, expression of the inhibitory ligand Qa-1 on $CD4^+$ T cells protect them from perforin mediated NK cell elimination following OVA immunization (Lu

et al., 2007). Moreover, NK cell depletion alleviate the immunopathology and subsequently impart the acute signature for the chronic viral infection (Waggoner *et al.*, 2011, Lang *et al.*, 2012). Intriguingly, regulatory T cells are prone to NK cell-mediated elimination in the manner that can be different from NK cells – mediated attack of CD4 or $CD8^+$ T cells (Roy *et al.*, 2008). Very recently, it has been reported that, liver NK (LrNK) cells inhibit the hepatic antiviral CD8 T cells in PD-1-PD-L1 dependent manner during adenovirus and LCMV infections (Zhou *et al.*, 2019).

In turn, T cells evolve different ways in order to guard themselves from NK cells jeopardy. For instance, NK cells which are devoid of inhibitory receptor 2B4 were shown to lyse activated T cells during LCMV infection (Waggoner *et al.*, 2010). Furthermore, two recent compelling studies have shown that type I IFN protect the T cells from NK cells –mediated attack in NCR1 and perforin mediated manner. In addition, type I IFN modulate the expression of inhibitory ligands which deemed to protect the activated T cells (Xu *et al.*, 2014, Crouse *et al.*, 2014).

Besides influencing T cells functions, the reciprocal engagement of NK cells with DCs result in paradoxical events. Of these, NK cells that express CCR7 induce the progression of DC maturation after migration to secondary lymphoid tissues. Subsequently, DCs secrete IL-12, IL-15, IL-18 and Type I Interferon after antigen uptake, which in turn calibrate the NK cells proliferation and lymphokines release (Moretta, 2002). Opposing to that, activated NK cells can eliminate MHC-I low expressing immature DCs (iDCs) through cell-contact interactions of NKp30 with its cognate ligand, to limit iDCs recruitment in inflamed tissues, other activating receptors like NKp46 have a very limited role here (Ferlazzo *et al.*, 2002). This elimination of immature DCs is noteworthy to curb the mature DCs supply, also the iDCs is a potent inducer for IL-10–producing regulatory T cells which counterparts the pathogen control (Jonuleit *et al.*, 2000). In addition to NK cell cytoxicity toward T cells and DCs in immunological synapse (Vivier *et al.*, 2008), the former could restrain hyperactive macrophages in mice and autologous activated macrophages in human, as evident from co-existence of NK cells in spleen and periphery as well as *ex vivo* settings, respectively (Gregoire *et al.*, 2007b, Nedvetzki *et al.*, 2007).

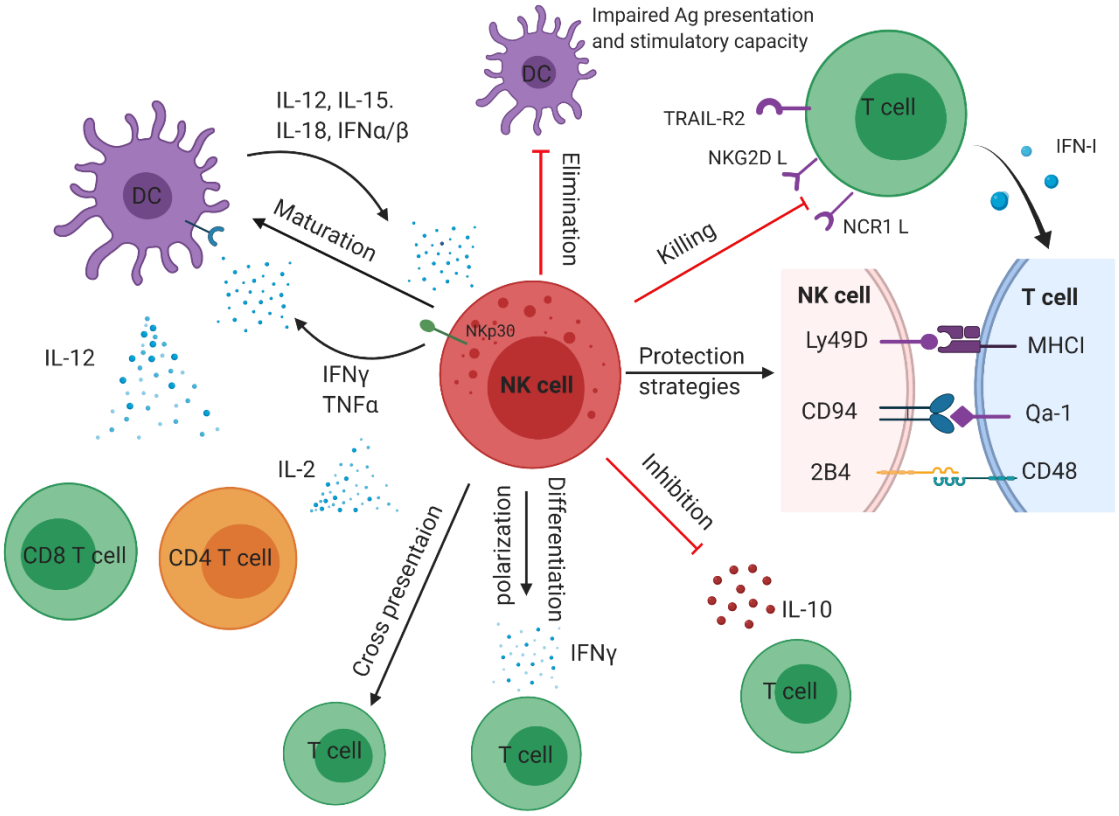

Figure 1.9: NK-DC-T cells crosstalk
Created with BioRender.com (by Thamer Hamdan, unpublished)

1.1.5 Viral Immunity

Viruses have distinct outcomes in the host immune system ranging from overzealous response to overt damage, and this outcome is determined by different factors such as; viral evading strategies, host genetic susceptibility, host age, dose and route of infection, as well as the balance between anti- and pro- inflammatory proteins. Viruses turn out in the host differently, some viruses induce the sterilizing cellular constituents of immune response then resolved without tissue damage like measles, mumps, rubella, and so. Some viruses cause persistent infection that is calibrated from the balance between immune response and viral load, or they can cause tissue damage due to their properties in compromising the efficacy of immune response like HIV, HCV, HBV as well as LCMV in murine models. In between, influenza and RSV generate variable response from mild/subclinical to fulminant disease. Further, viruses like EBV and coxsackie B virus triggers autoimmune diseases and cancer. Hosts have many factors to limit the tissue damage, of these; anti-inflammatory cytokines

like IL-10, TGFB, innate immune regulatory receptors, chemical mediators as well as, Treg cells. For instance, binding of inhibitory receptors on effector T cells to their cognate ligands will transmit inhibitory signals to effector T cells and hence control the inflammatory activity and subsequent cell damage. Moreover, Treg cells or highly polarized effector T cells can produce anti-inflammatory cytokines like IL-10 and TGF-β that inhibit effector cells. Further, the balance between the aforementioned anti-inflammatory and pro-inflammatory mechanisms determine the outcome of viral infection. Of these, pro-inflammatory signals are; chemical mediators, proteinases, reactive species, IL-2, IFNs and other chemokines. If the pro-inflammatory signals outpace the anti-inflammatory ones, this will confine the viral load and exacerbate the tissue damage, nevertheless, the opposite scenario result in virus persistence in the host as subclinical or opportunistic (Rouse and Sehrawat, 2010, Welsh and Waggoner, 2013).

After entry of viruses into host cells, the cytopathic viruses (VSV in mice and rabies or smallpox in human), or non-cytopathic viruses breach the first line defence, they replicate at the site of infection. The cytopathic viruses kill infected cells causing the release of cellular contents, including proteases and lysosomal enzymes, which digest the extracellular matrix and create an inflammatory milieu. Recruited neutrophils release inflammatory mediators. Beside, innate cells recognize viral replication intermediates and secrete pro-inflammatory cytokines, which clear the virus and contribute to tissue damage. Viral antigens are taken up by antigen-presenting cells and carried to local draining lymph nodes. Based on the cytokine milieu created in the draining lymph node, different types of T_h cell responses are induced. Primed CTLs migrate to the site of infection and kill virally infected cells, thereby contributing to tissue damage. After migrating to the site of infection, T_h cells also contribute to the tissue damage. In conditions in which the control of aggressive T_h cells and CTLs by regulatory T cells is impaired and other inhibitory pathways fail to curtail them, tissue damage is the main consequence of viral infection. T_h cells also provide help to B cells to secrete antibodies, which form immune complexes that are deposited in certain tissues such as the glomeruli of the kidneys and blood vessels to cause immune complex-mediated disease (Rouse and Sehrawat, 2010, Hangartner *et al.*, 2006).

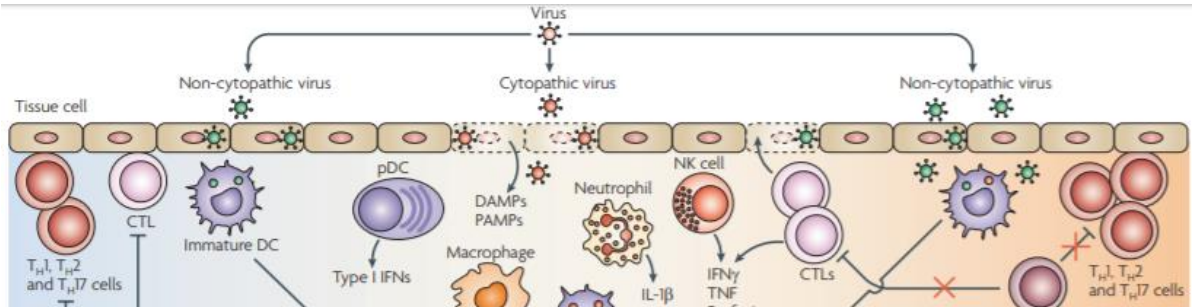

Figure 1.10: Viral infection outcomes
Rouse BT and Sehrawat S (2010): Immunity and immunopathology to viruses: what decides the outcome?. Nat Rev Immunol

1.2 Fc Receptors (FcRs)

The receptors of the fragment crystallisable (Fc) portion of immunoglobulin (Fc receptors, FcRs) are ubiquitously found on a variety of cell types of the immune system with versatile functions. They belong to the large immunoglobulin (Ig) superfamily, and are type I transmembrane glycoproteins (the carboxyl terminus of the polypeptide chain is located in the cytosol, whereas the amino terminus is exposed to the extracellular space) (Nimmerjahn and Ravetch, 2008, Gessner *et al.*, 1998).

On the basis of the type of antibody they recognise, FcRs are mainly classified as FcαR, which binds to IgA; FcδR, which binds to IgD; FcεR, which binds to IgE; FcγR and neonatal Fc receptor, which binds to IgG; and FcμR, which binds to IgM (Fridman, 1991, Pyzik *et*

al., 2019). FcγRs perform a wide range of functions posed on afferent and efferent immunity. In addition to their essential role in specific binding to the Fc portion of antibody subsets, their binding to immune complexes on dendritic cells (DCs) and macrophages (Mφ) leads to phagocytosis and presentation of antigenic peptides via major histocompatibility complex (MHC) class I and class II proteins . These proteins are further recognized by T-cell subsets, resulting in the activation of these cells and mediating their functions accordingly (Guilliams *et al.*, 2014, Sun, 2014).

Generally, FcRs are among the immune receptors that recognize antigens indirectly along with B-cell receptors (BCRs) and T-cell receptors (TCRs), these receptors have activation motifs and signalling pathways in common. FcRs recognize Fc portion of antibody rather than antigens directly, forming a complex of membrane- bound receptors for antigens. Several factors dictate the FcR–antibody engagement outcomes that affect the expression levels of activating and inhibitory FcγRs by the virtue of cytokines or alteration in the affinity of the antibody–FcγR binding due to differential antibody glycosylation. FcRs prevail in two forms; membrane receptors and soluble molecules, generated by alternative splicing of FcR transcripts or by proteolysis of membrane receptors and have a distinct role in B cells proliferation and antibody production (Daeron, 1997, Nimmerjahn and Ravetch, 2008, Fridman *et al.*, 1993, Hamdan *et al.*, 2020).

1.2.1 The γ subunit of immunoglobulin Fc receptor (FcRγ)

The γ subunit of the immunoglobulin Fc receptor (FcRγ) is a salient homodimeric part of various Fc receptors, namely the high-affinity receptor for IgE (FcεRI in mice and humans), the high-affinity receptor for IgG (FcγRI or CD64 in mice and humans), the low- to medium-affinity receptor for IgG (FcγRIII or CD16 in mice and FcγRIIIA in humans), the low-affinity receptor for IgA (FcαRI or CD89 in humans). Moreover, Fcγ associates as a heterodimeric unit with various immunoreceptors, such as NKp46; the platelet collagen receptor glycoprotein VI; the paired immunoglobulin-like receptor A (PIR-A), which is homologous to human CD85; the interleukin (IL)-3 receptor; the osteoclast-associated receptor (OSCAR); signal-regulatory protein β1 (SIRPβ1); triggering receptor expressed on myeloid cells (TREM) and Dectin-1 (Hida *et al.*, 2009, Nimmerjahn and Ravetch, 2008, Takai *et al.*, 1994, Duhan *et al.*, 2019, Brandsma *et al.*, 2016). Collectively, the γ subunit is indispensable not only as a structural part of Fc receptors but also as a pivotal member of an array of immune functions, including signal transduction (Takai *et al.*, 1994). In this context,

FcRγ is also called FcεRIγ because FcRγ was first noticed as the third subunit of FcεRIγ and subsequently as a common gamma chain on FcγRIII (CD16), FcγRI (CD64), and FcαRI (CD89) (Kuster *et al.*, 1990, Brandsma *et al.*, 2016, Suzuki *et al.*, 1998, Hamdan *et al.*, 2020).

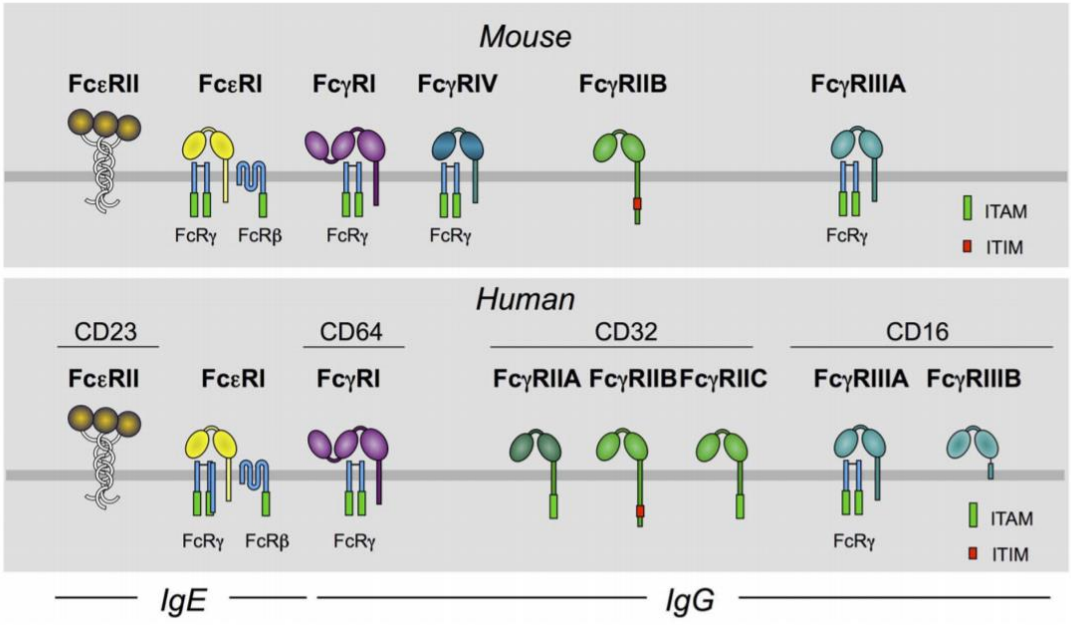

Figure 1.11: Mouse and human receptors for the Fc portion (FcR) of IgE and IgG
Jönsson F *et al.*, (2012): Mast cells and company. Front Immunol

1.2.2 Biological functions of FcγRs

The FcγR family performs paradoxical functions as activators or inhibitors because of their structural heterogeneity and are involved in regulating various immune responses. Along with their essential role in specific binding to the Fc portion of antibody subsets, FcγRs calibrate multiple effector responses, such as antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), release of inflammatory substances, B cells activation, DCs maturation, antigen presentation, the release of inflammatory substances and macrophage polarization (Nimmerjahn and Ravetch, 2008, Nimmerjahn and Ravetch, 2006, Bianchini *et al.*, 2019, Sironi *et al.*, 2006, Swisher *et al.*, 2014).

Generally, Fc receptors perform three main functions. First, they up- or down regulate immune-cell responses, such as the proliferation of B cells, phagocytosis by macrophages, and degranulation of mast cells as well as down modulation of immune responses. Second,

they take up immune complexes (ICs) by trigger the phagocytosis of captured immune complexes (ICs) after its uptake, which leads to eradication of the antigen–antibody complexes and delivering s the antigenic peptides to the MHC class I or class II antigen–presentation pathway (Amigorena and Bonnerot, 1999). Third, they perform non-immunoregulatory functions: the neonatal FcR of IgG (FcRn), which is responsible for the vertical transfer of maternal IgG, and the polymeric immunoglobulin receptor (poly-IgR), which is responsible for the transfer of IgA to mucosal surfaces (Takai, 2002, Hamdan *et al.*, 2020).

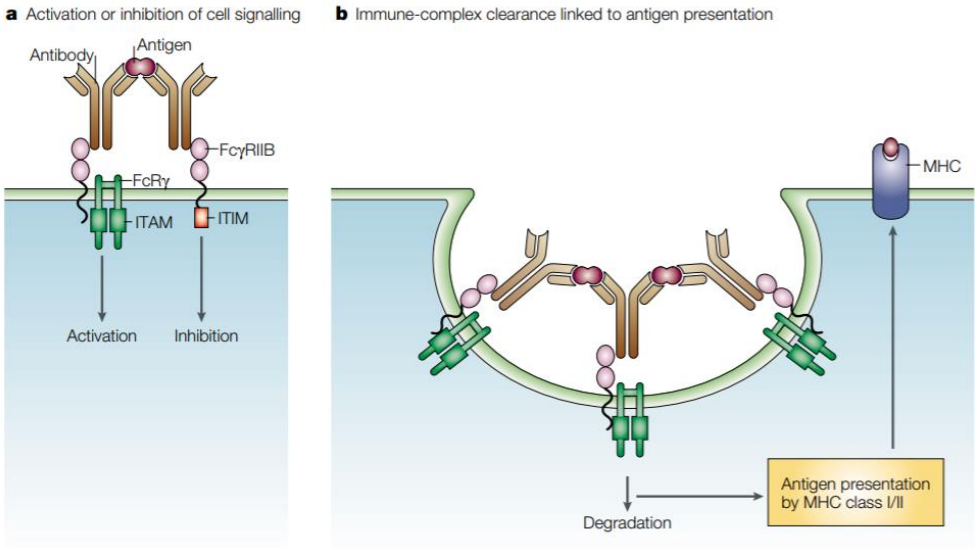

Figure 1.12: Major functions of Fc receptors
Takai T (2002): Roles of Fc receptors in autoimmunity. Nat Rev Immunol.

1.2.3 FcγRs signalling pathways

Fc receptors exist as activation receptors and relay their signals via ITAM, and as inhibitory receptors, which transmit their signals via the immunoreceptor tyrosine-based inhibitory motif (ITIM) (Nimmerjahn and Ravetch, 2006, Ravetch and Lanier, 2000). The activation of FcγRs requires a ligand-binding α-chain adaptor molecule containing ITAMs that are extended in its cytoplasmic domain to activate signalling pathways. Structurally,

adaptor proteins differ among immune cells. For example, FcγRIIIA on NK cells is associated with T-cell receptor z-chain (Li and Kimberly, 2014), yet FcγRIIIA associates with the common γ-chain in monocytes and macrophages. Furthermore, FcεRI and the FcγRIII in basophils and mast cells has an extra β-chain (Maeda *et al.*, 2003). Upon crosslinking with immune complexes, the signalling pathway begins with tyrosine phosphorylation of the ITAMs linked to the γ chain via SRC family kinases (SFKs) such as; Fyn and Lyn. These ITAMs form docking sites for SYK family kinases, culminating in the subsequent recruitment of other downstream proteins, such as phosphoinositide 3-kinase (PI3K), which activate downstream kinases and lead to the release of calcium from the endoplasmic reticulum (ER) and ensuing in the release of granular content, which include perforin and granzymes (Ghazizadeh *et al.*, 1994, Wang *et al.*, 1994). Notwithstanding, the inhibitory FcγRIIB transduces inhibitory signals by phosphorylating the tyrosines present in ITIMs. This inhibition requires the ligation between activating heterologous receptor (e.g., the BCR) and the inhibitory FcR that is mediated by immune complexes. Following phosphorylation of tyrosines in ITIM by Lyn, SH2-containing inositol 5′-Phosphatase (SHIP) are recruited and phosphorylated. The SRC phosphatase, SHIP1/2, regulates cellular levels of PI(3,4,5)P3 by hydrolyzing it PI(3,4)P2 and this dephosphorylation inhibits cell proliferation. ITAM motifs are not acting always as activating but also as inhibitory, called ITAM-mediated inhibitory signal, ITAMi. Unlike the ITIM, ITAMi does not require co ligation with heterologous receptors, but the inhibitory signals. The Bi-functionality of ITAM is key to ensure the immune maintenance and to reduce the autoimmune diseases development. ITAMi is activated following interaction with low affinity avidity such as; such as FcαRI, FcγRIIA and FcγRIIIA. Upon binding of monomeric immunoglobulin to FcR bearing ITAM motif (e.g.,FcγRIIA), the last tyrosine residue of the ITAM motif by is phosphorylated by Lyn responsible for transient recruitment of Syk followed by that of SHP-1 which halts the activation signal (Ben Mkaddem *et al.*, 2019, Bolland and Ravetch, 1999, Rohrschneider *et al.*, 2000, Hamdan *et al.*, 2020).

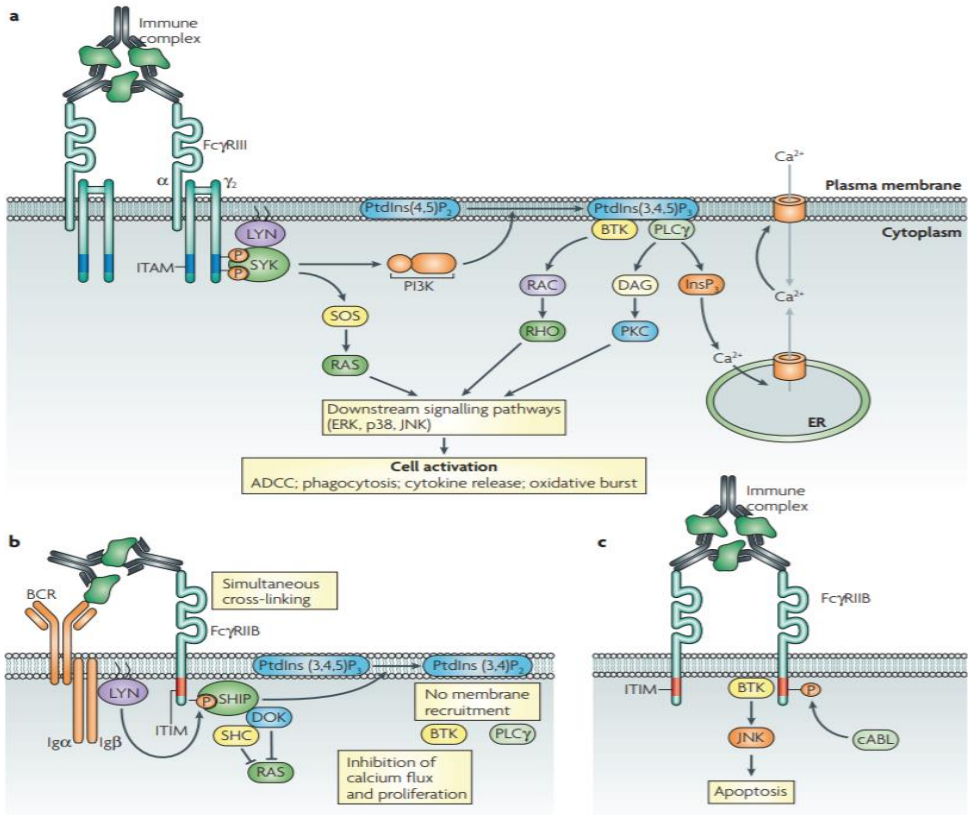

Figure 1.13: FcγRs signalling pathways
Nimmerjahn F *et al.*, (2008): Fcgamma receptors as regulators of immune responses. Nat Rev Immunol.

1.2.4 Structure of FcRs

The conformational states of the Fc domain determine the identity of FcRs, two distinct sets of FcRs have been identified for IgG: the canonical type I FcRs bind the open conformation of Fc domain which belong to the Ig receptor superfamily and include the activating receptors FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa, and FcγRIIIb; and the inhibitory receptor FcγRIIb. On the other hand, type II FcRs are represented by the family of C-type lectin receptors, which includes CD209 (DC-SIGN; homologous to SIGN-R1 in mice) and CD23. Along with the Fab portion, this heterogeneity of effector molecules that are engaged by the Fc domain imparts polyclonality to antibodies (Pincetic *et al.*, 2014, Bournazos *et al.*, 2017). The high-affinity FcγRIs (FcγRIA, FcγRIB, and FcγRIC) are encoded by a single mouse gene but by three human genes. Moreover, the low-affinity FcγRs (FcγRIIs, such as FcγRIIA, FcγRIIB, and FcγRIIC; and FcγRIIIs, such as FcγRIIIA and FcγRIIIB) are encoded by two mouse genes but by five human genes. FcεRI is a tetramer consisting of one alpha chain, one beta chain, and two gamma chains. These gamma chains are also a shared

subunit of other Fc receptors and are encoded by the fcer1g gene, which contains five exons and spans four kilobases. This gene evolved from the same ancestor as the zeta chain of T-cell receptors; both of these genes are located on the long arm of chromosome 1 (1*q*21–23) (Bournazos *et al.*, 2016, Qiu *et al.*, 1990, Kuster *et al.*, 1990, Li and Kimberly, 2014, Hamdan *et al.*, 2020).

1.2.5 Therapeutic approaches of FcRs

FcRs are the mainstay regulators of the immune system by virtue of their ability to calibrate tolerance and prime the effector functions of humoral and cellular components. Indeed, targeting these adaptor proteins exerts a promising impact on immunotherapeutic settings. Compelling evidence from several studies emphasizes the importance of inhibitory FcγRIIB in maintaining peripheral tolerance (Li *et al.*, 2014) and humoral tolerance (Baerenwaldt *et al.*, 2011). In case of imbalance between activating and inhibitory FcRs, a state of IC-triggered autoimmune diseases is ensued as the activation of FcRs primes the autoimmune pathology, and functional restoration of inhibitory FcγRIIB in humans and mice by an agonist ameliorates these diseases (McGaha *et al.*, 2005), also downmodulation or neutralising the FcγRs interactions with immune complexes via an antagonist preclude the IC-mediated inflammatory responses that are driven by autoantibodies (Clynes *et al.*, 1999). The reverse scenario is also plausible: amplifying the activity of antibodies by enhancing their interaction with activating FcγRs which are a mediators of ADCC, or blocking antibody binding to the inhibitory receptor, and hence amplifying ADCC reactions in immunotherapeutic approaches where the autoreactive B cells has a detrimental role in autoimmune injury. Furthermore, FcRs may be targeted either with variant antibodies to facilitate the relay of antigens into DCs for antigen presentation or alteration the Fc subunit of therapeutic antibodies at post-translational level (Nimmerjahn and Ravetch, 2008, Takai, 2002, Li and Kimberly, 2014, Hamdan *et al.*, 2020).

1.3 Lymphocytic choriomeningitis virus (LCMV)

1.3.1 Biological background

Lymphocytic choriomeningitis virus (LCMV) is a prototypical member of *Arenaviridae*, which include also other human germs such as *Lassa virus* (Oldstone *et al.*, 1985). They gain their name from *arenosus*, the Latin word for "sandy," based on their fine granularities appearance within the virion (Bonthius, 2012). LCMV is a non-cytopathic virus replicating in its natural host and reservoir, which is the house mouse, *Mus musculus*, and the human along with other animals like hamsters. In human, some LCMV strain causes substantial neurological problems (Biggar *et al.*, 1975). LCMV is one of the widely used model systems to study viral persistence and pathogenesis depending on the virus strain and dose (Welsh and Seedhom, 2008). It is an enveloped ambisense RNA virus and consists of glycoprotein (GP) spikes on the surface and two negative sense-single stranded RNA segments named L and S, which are wrapped along nucleoproteins (Lee *et al.*, 2000). LCMV infects cells by binding through their glycoprotein to cell surface receptor called alpha-dystroglycan (α-DG), which is expressed in a company with extracellular matrix (ECM) components such as; stromal and epithelial cells in many tissues, as well as on dendritic cells (Cao *et al.*, 1998). Upon receptor binding LCMV is internalized in to cell vesicle where viral membrane fuse to cell membrane and release its genome in cytoplasm (Meyer *et al.*, 2002). Historically, LCMV was first described by Charles Armstrong in 1933 during epidemic encephalitis in St. Louis (Zhou *et al.*, 2012).

1.3.2 LCMV strains

LCMV strains are classified into; the Armstrong strain which was isolated from a monkey with a lymphocytic choriomeningitis. The Traub strain was obtained from a laboratory colony of persistently infected mice. The WE strain was isolated from a human after exposure to persistently infected mice (Welsh and Seedhom, 2008). Other LCMV strains originated from these 3 isolates. For instance, Aggressive and Docile strains are clones of strain WE, and clone 13 was derived from strain Armstrong with a difference in two nucleotides (Takagi *et al.*, 2012). In terms of the immune response, LCMV strains can induce a chronic or an acute infection. Armstrong and WE induce an acute infection in mice; the virus can be cleared 8 days *post-infection*. Chronic infections can result from Clone 13 and docile strain. Furthermore, LCMV CL-13 is more infectious than Armstrong strain due to higher affinity for α-DG and widely spread tissue tropism (Mueller and Ahmed, 2009)

1.3.3 LCMV pathogenesis

Different strains of LCMV result in versatile outcomes in terms of T cell responses and virus control. WE and Armstrong infection induce acute infection where the viral particles are resolved within few days *post infection*. Unlike, docile and clone 13 strains cause chronic infection and it can take up to few weeks to clear the viral particles. Mice infected with lower dose such as 200 Plaque forming units (PFU) of LCMV-Docile cleared virus within 2-4 weeks because of normal cytotoxic T cell response (CTL), whereas mice infected with higher doses (1×10^7 PFU) generate less pronounced CTL responses and the viral particles are detectable until day 280 post infection (Moskophidis *et al.*, 1993). On the other hand, mice infected with intermediate dose of 1×10^4 PFU of LCMV-Docile fail to clear the virus and exhibit overwhelming immunopathology. The balance between the viral load and T cell response is depicted below (Fig 1.14). Exhaustion is a common phenomenon in the context of chronic infection where the T-cells lose their ability to produce cytokines such as IL-2, also the high proliferative capacity and *ex vivo* killing are lost. The production of antiviral cytokines such as TNF-α and IFN-γ are abrogated, followed by physical deletion of $CD8^+$ T cells (Wherry, 2011). High viral load drives T cell exhaustion in the course of chronic viral infection (Mueller and Ahmed, 2009). Moreover, the virus-infected hepatocytes are recognised by $CD8^+$ T cells killing which results in fulminant immunopathology and liver damage (Maini *et al.*, 2000).

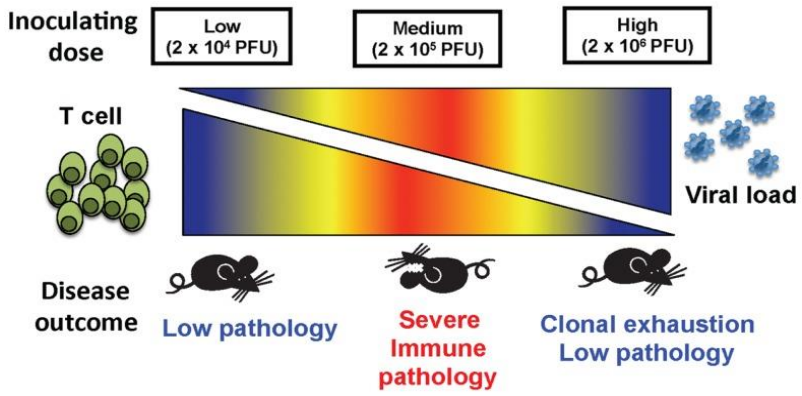

Fig 1.14: impact of viral load on the T cells response
Cornberg M *et al.*, (2013). Clonal Exhaustion as a Mechanism to Protect against Severe Immunopathology and Death from an Overwhelming $CD8^+$ T cell Response. Front Immunol.

2 AIMS AND OBJECTIVES

'Physical embrace' between constituents of innate and adaptive arms of the immune system has a profound impact on the ensuing immune response. Of these, NK cells, DCs and $CD8^+$ T cells exquisite interactions are deemed as bridge between the innate and adaptive immune systems. Due to the paucity of observations regarding the molecules which modulate this circuit between NK cells and cytotoxic T cells upon viral infections, we intended to delineate the impact of the innate sentinels represented by NK cells on the antiviral effector cytotoxic T cells during the context of chronic viral infections in the murine model. To that aim, we used the prototypic LCMV virus that is NK cells resistant but have a demonstrable effect upon T cells response. In pursuit of our objective, we exploited FcRγ, FcεRIγ, *Fcer1g*$^{-/-}$ mice that lack the Fc-receptor common gamma chain to dictate the role of Fc-receptor and NK-receptor signalling in the process of $CD8^+$ T cell regulation.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Reagents

Chemicals were obtained from AppliChem (Darmstadt), Merck (Darmstadt), Roth (Karlsruhe) or Sigma-Aldrich (Munich).

Reagent	**Supplier**
Fetal Calf Serum (FCS)	Biochrom AG Berlin
Isofluran	Delta Select GmbH, Pfullingen
Carboxyfluorescein Succinimidyl Ester (CFSE)	Invitrogen, Darmstadt
RBCs lysing solution	BD Bioscience, Heidelberg
RNeasy Mini Kit	Qiagen, Hilden, Germany
QuantiTect Reverse Transcription Kit	Qiagen, Hilden, Germany
SYBR®Green	AppliedBiosystems, Darmstadt
TaqMan Fast Universal PCR Master Mix (2X)	AppliedBiosystems, Darmstadt
Mouse NK cell isolation kit	Milteny Biotec, Germany
MG-132	Enzo Life Sciences, USA
Mouse IL-2	Milteny Biotec, Germany
2-β mercaptoethanol	Sigma, Munich
Brefeldin A	eBioscience
Saponin	Sigma, Munich
o-Phenylenediamine dihydrochloride	Sigma, Munich
Formaldehyde, Acetone, Methanol, Isopropanol	Sigma, Munich
Ethanol	Merck
Ethylenediaminetetracetic acid (EDTA)	Sigma, Munich
L-Glutamine–Penicillin–Streptomycin solution	Sigma, Munich

3.1.2 Media and Solutions

Name	Supplier/Preparation
Dulbecco's Modified Eagle's Medium (DMEM)	PAN Biotech GmBH, Aldenbach
DMEM medium For cell culture	10 (2%), 25 (5%) or 50 mL (10%) FBS superior (FCS), 5 mL Glutamine- Penicillin-Streptomycin solution
DPBS w/o Mg^{2+}, Ca^{2+}	PAN Biotech GmBH, Aldenbach
DEPC-treated water	Ambion, ThermoFisher Scientific
RPMI 1640	Merck, Germany
NK cells culture medium	RMPI 1640 with 20mM HEPES w/o $NAHCO_3$+10%FCS+50µM2-Mercaptoethanol+ penicillin, streptomycin and L-glutamine)
Tissue-Tek O.C.T. Compound	Sakura
10x FACS-Buffer (IL)	50 ml –0.5M EDTA 10gm NaN_3 100ml FCS 100gm PBS 800ml dd H_2O
Overlay (Plaque Assay)	50% (v/v) IMDM Solution 50% (v/v) 2% Methylcellulose
Fluorescent Mounting medium	DAKO
MACS Buffer	0.5% FCS, 2mM EDTA in PBS For 100ml- 100ml PBS+ 500ul FCS + 400ul 0.5M EDTA

3.1.3 Primers

Name	Supplier
Fcer1g Mutant	CTCGTG CTT TAC GGT ATC GCC (Biomeres)
Fcer1g Common	ACC CTA CTC TAC TGT CGA CTC AAG
Fcer1g WT	CTC ACG GCT GGC TAT AGC TGC CTT
GAPDH Mm_Gapdh_3_SG	QuantiTect Primer Assay (200), Quiagen GmbH, Hilden
CD247 (CD3ζ)	Mm00446171_m1, Thermofisher
Mm_Ncr1_1_SG QuantiTect Primer Assay	Qiagen GmbH, Hilden

3.1.4 Equipment

Equipment	Supplier
BD LSRFortessa™ cell analyzer	BD Bioscience
Fluorescent -Microscope HS BZ-9000	Keyence GmbH, Neu-Isenburg
Cryostat CM 3050S	Leica, Wetzler
NanoDrop ND-1000	Peqlab Biotechnologie GmbH, Erlangen
TissueLyserII	QIAGEN, Hilden
Micro 220R-Centrifuge	Andreas Hettich GmbH, Tuttlingen
LightCycler 480 realtime PCR machine	Roche
Thermocycler PCR machine	Applied Biosystems
Micro-centrifuge	Hettich
Centrifuge 2210R	Eppendorf

3.1.5 Software

Name	Supplier
FacsDiva v&6.2.1	BD Bioscience
FlowJo V10	Tree Star Inc., Ashland (OR, USA)
Graph Pad Prism, version 7.03	GraphPad Software,USA
Microsoft Office 2007	Microsoft Corporation, Redmond (WA, USA)
BioRender illustrations	www.biorender.com

3.1.6 Antibodies

The information for all antibodies used for this study is provided in a supplementary table.

3.1.7 Murine Models

All mice used in these studies were maintained on the C57BL/6 (B6) genetic background unless stated otherwise. Besides the C57BL/6 (B6) mice which were used as wild type (WT) mice along with littermates, different mouse strains have been used in these experiments.

3.1.7.1 *Fcer1g*$^{-/-}$ mice

These mice are deficient in the gamma chain subunit of the FcgRI, FcgRIII, and FceRI receptors. Different immune cells including the NK cells are functionally impaired, due to the lack of these Fc receptors (Takai *et al.*, 1994). Those mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA; stock# 002847) and were maintained on a mixed (B6; 129P2) background. Littermate WT or heterozygous mice (*Fcer1g*$^{+/-}$) served as controls for all experiments.

3.1.7.2 P14 mice

These transgenic mice have T-cell receptor (TCR) specific for the LCMV glycoprotein epitope 33 (GP-33-41) presented on MHC class I (Pircher *et al.*, 1989). P14 mice are crossed with CD45.1 congenic mice to track lymphocytes during adoptive transfer experiments.

3.1.7.3 *Jh*$^{-/-}$ mice

These mice are devoid of genes for heavy chain joining region (JH segment) and intron enhancer and thereby fail to develop functional B-cells (Chen *et al.*, 1993).

3.1.7.4 *Ifnar*$^{-/-}$ mice

These mice lack the receptors for interferon a and b and thus IFN signalling, and are highly prone to viral infection (Muller *et al.*, 1994). Further, *Ifnar*$^{-/-}$ *P14* mice expressing congenic markers CD90.1 were used track lymphocytes during adoptive transfer experiments.

3.1.8 Lymphocytic Choriomeningitis Virus (LCMV)

The LCMV-WE strain was originally obtained from F. Lehmann-Grube (Heinrich Pette Institute, Hamburg, Germany) and was propagated on L929 cells (obtained from ATCC, NCTC clone 929). LCMV-Docile was provided by Prof. Dr. R. Zinkernagel (University of Zurich, Zurich, Switzerland) and was propagated on L929 cells. Mice were infected intravenously with 2 x 10^4 plaque-forming units (PFU) for Docile LCMV and with 200 plaque-forming units (PFU) for WE LCMV.

3.1.9 Ethics statement

Animal experiments were authorized by the Veterinäramt Nordrhein-Westfalen (Düsseldorf-Essen, Germany) and were conducted in accordance with the German laws for animal protection or according to institutional guidelines at the Ontario Cancer Institute of the University of Toronto Health Network. All animal experiments were approved under license number 84-02-04.2013.A242 by Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen. All animal care and use protocols adhere to national (Tierschutzgesetz) and European (Directive 2010/63/EU) laws and regulations as well as European Federation of Animal Science Associations (FELASA) http://www.felasa.eu/. Animals were euthanized using the cervical dislocation method.

3.2 Methods

3.2.1 Plaque assay

Mice were put to death at different time as mentioned in the figure legends, and LCMV viral titers were detected on MC57 fibroblasts (provided by the Ontario Cancer Institute (Toronto, ON, Canada)) by a plaque-forming assay, as previously described (Battegay *et al.*, 1991). Briefly, organs were smashed in Dulbecco's modified Eagle medium (DMEM) containing 2% fetal calf serum (FCS), titrated 1:3 over 12 steps in 96-well round-bottom plates, and plaqued onto MC57 cells. After incubation for 3 h at 37°C, an overlay was added (1:1 mixture of methyl cellulose and Iscove's Modified Dulbecco's Medium), and the virus preparation was again incubated at 37°C. Plaques were stained 48 hours later. For staining, cells were fixed with 4% formaldehyde solution in phosphate-buffered saline (PBS), permeabilized with a 1% Triton-X solution in PBS, blocked with 10% FCS in PBS, and

stained with anti-LCMV nucleoprotein (NP) antibody (made in house). Enhanced chemiluminescence (ECL)-conjugated anti-rabbit IgG antibody was used as a secondary antibody. Plaques were detected by color reaction (0.2 M Na_2HPO_4 + 0.1 M citric acid + 30% H_2O_2 + o-phenylenediamine dihydrochloride). All chemicals were purchased from Sigma-Aldrich (Germany).

3.2.2 Tetramer, surface, intracellular Staining, and flow cytometry analysis

All of the antibodies used in this study are listed in S1 Table. The LCMV-specific $CD8^+$ T cell response upon LCMV infection was measured with a tetramer complex of major histocompatibility complex (MHC) class I ($H\text{-}2D^b$) and LCMV $GP_{33\text{-}41}$ (KAVYNFATM) peptide. Tetramers were provided by the National Institutes of Health (NIH) Tetramer Facility (Bethdsda, MD, USA). Cells were stained with allophycocyanin (APC)-labeled GP33 MHC class I tetramer ($GP33/H\text{-}2D^b$) for 15 minutes at 37°C. After incubation, the samples were stained with monoclonal antibody to CD8a for 30 minutes at 4°C. Absolute numbers of GP33-specific $CD8^+$ T cells were counted by fluorescence-activated cell sorting (FACS) with calibrating beads (340486; BD Bioscience, Germany). For Lamp-1 staining, anti-CD107a antibody was added for 5hrs incubation period, and the immunofluorescence was measured after additional staining with anti-NK1.1 antibody. For intracellular cytokine staining, smashed splenocytes from LCMV-infected mice were cultured in 5% FCS DMEM medium supplemented with LCMV $GP_{33\text{-}41}$ peptide (5 µg/ml) for 1 hour at 37°C in an incubator. After 1 hour, brefeldin A (25 µg/ml; B7651; Sigma, Germany) was added, and the cells were incubated for another 4 hours at 37°C. After a total of 5 hours, splenocytes were washed with FACS buffer, stained for surface anti-mouse CD8 antibody at 4°C for 30 minutes and then fixed with 2% formalin in PBS at room temperature for 10 minutes. After another washing step, cells were incubated for intracellular staining with antibodies to IFN-γ and TNF-α in 0.1% saponin (S4521; Sigma) in FACS buffer for 30 minutes at 4°C, washed, and analyzed with flow cytometry.

3.2.3 NKp46-ligand detection *in vitro*

For NKp46-ligand expression on LCMV–specific $CD8^+$ T-cells (P14 * 45.1), recombinant mouse NKp46/NCR1 Fc Chimera Protein, which is a fused murine NKp46 and human IgG1 Fc region (2225-NK-050; R&D systems) was used. $1*10^6$ splenic cells were incubated with 1ug of this chimeric protein for 2 hours at 4°C, washed and then stained with human IgG Fc

APC-conjugated Antibody (FAB110A; R&D systems) as a secondary antibody for 30 minutes and analyzed by flow cytometry. As negative control, Recombinant Human IgG1 Fc, CF (110-HG-100) was used.

3.2.4 RNA isolation and quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from NK cells with the RNeasy Mini Kit (Qiagen, Hilden, Germany). For RNA quantification, a NanoDrop ND-1000 spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany) was used. Complementary DNA synthesis was performed with the QuantiTect Reverse Transcription Kit *(*Qiagen). For quantitative reverse transcription polymerase chain reaction (qRT-PCR) either the Fast SYBR Green Master Mix (Applied Biosystems, Darmstadt, Germany) or the TaqMan Fast Universal PCR Master Mix (2X; Applied Biosystems) was used on a 7500 Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). Primers for *NCR1, Fcer1g and CD247* were purchased from Qiagen or Thermofisher (Germany). For analysis, the expression levels of all target genes were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ΔCt). Gene expression values were calculated with the ΔΔCt method.

3.2.5 YAC-1 *in vitro* killer assay

Splenic NK cells were obtained from donor mice (WT and *Fcer1g*$^{-/-}$) 2 days after Docile strain of LCMV infection using murine NK cells isolation kit (Miltenyi Biotech, Germany). 20.000 YAC-1 cells were co-cultured with NK cells in triplicates in a ratio of 1:1, 1:10 and 1:25. After 4 h incubation at 37°C, surface stainings were performed in PBS and the samples were washed. The apoptotic markers, Annexin V (Biolegend) and 7AAD (Biolegend) were diluted in Annexin V Buffer (BD Biosciences) and incubated for 30 min at 4°C followed by acquisition of the samples.

3.2.6 *In vivo* killer assay

10^6 negatively MACS sorted CD8$^+$ T cells from spleens of P14 x CD45.1 mice were adoptively transferred into *Fcer1g*$^{-/-}$ mice and on next day, those mice were infected i.v. with 200 PFU of LCMV-WE strain. After 5 days, negatively MACS sorted total CD8$^+$ T cells (1 x 10^6 cells per mouse) were transferred into NK cell depleted naïve C57BL/6 mice or WT and *Fcer1g*$^{-/-}$ mice which were already i.v infected with 200 PFU of LCMV-WE 3

days before. After 4 hours of *in-vivo* incubation in recipient mice, spleens were harvested and the total number of P14 cells were analyzed and calculated by FACS.

3.2.7 Lymphocyte adoptive transfer

Splenocytes or negatively sorted $CD8^+$ T cells from P14 ($CD45.1^+$) or *IFNAR*$^{-/-}$ × *P14* ($CD90.1^+$) mice were injected intravenously into mice of interest. One day later, mice were infected with LCMV-docile strain. To identify the proliferation of transferred cells, splenocytes from P14/CD45.1 mice were stained with 1 µM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, Germany) in PBS for 10 minutes at 37°C, washed 2 times with 10% FCS DMEM media, suspended in plain DMEM media and injected intravenously into mice. One day later, mice were infected with LCMV-WE and the proliferation of P14 T cells in the spleen was assessed with CFSE dilution by flow cytometry.

3.2.8 Liver enzyme activity measurements

The activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) was measured in the Central Laboratory, University Hospital Essen, Germany.

3.2.9 Purification and culture of NK cells

NK cells were negatively sorted with a mouse NK cell isolation kit (130-115-818; Milteny Biotec, Germany) according to the manufacturer's protocol. For NK cultures, sorted NK cells were stimulated with 1,000 U/ml IL-2 (Miltenyi) for 2 days and were then treated with 20µg/ml of MG-132 (a proteasome inhibitor) purchased from (Enzo Life Sciences, Farmingdale, NY, USA; BML-Pl102-0025).

3.2.10 NK cell depletion

NK cells were depleted with an intraperitoneal injection of anti-NK1.1 (clone PK136 from Bioxcell; 200 µg per mouse) or mouse IgG2a isotype control (from Bioxcell) on day 3 before infection and on day one after infection, as previously described (Lang *et al.*, 2012).

3.2.11 Histology

Histologic analyses of snap-frozen tissue were performed with a mAb to anti-CD8a-PE, and mouse monoclonal antibodies to LCMV nucleoprotein (NP; made in house). In short,

sections were fixed with acetone for 10 min, and nonspecific antigen binding was blocked in PBS containing 2% FCS for 15 min, followed by staining with various antibodies for 45 min. All antibodies were diluted 1:100 from their original concentration in blocking solution. Images of stained sections were acquired with a fluorescence microscope (KEYENCE BZ II analyzer; KEYENCE Corporation of America, Itasca, IL, USA).

3.2.12 FACS analysis

FACS acquisition was performed on BD FACS Fortessa and analysis was performed on FlowJo 10. Organs were harvested and then crushed in cold PBS. Cells were collected after washing with PBS once in a BD FACS tube. Antibody cocktail was made with desired antibodies in FACS buffer in a dilution of 1:100/ sample. Samples were then incubated at 4 °C for 30 min and then washed with FACS buffer via centrifugation at 1500rpm for 5 min. Cells were re-suspended in FACS buffer and were analyzed.

3.2.13 Virus-Specific $CD8^+$ T cell staining.

Samples were taken in FACS tubes and were incubated with fluorescent Labelled (APC) GP33 tetramer for 15 min at 37°C. Cocktail of CD8 along with other desired markers was made in FACS buffer as 1:100/sample and added to each sample. Samples were then incubated at 4°C for 30 min and then washed afterwards with FACS buffer. In case of blood samples, erythrocytes were lysed with BD Cell Lysing buffer for 7 min and then washed with FACS buffer. Samples were then re-suspended in FACS buffer and were analyzed.

3.2.14 Mice genotyping

To determine the transgenic state of the mice, genotyping was performed. Mice were ear-marked and 2-4 mm of tail was cut and put into 600 µl digestion buffer. Proteinase-K was then added at the concentration of 20 ug/ml. Samples were then incubated at 56°C till the tails were dissolved properly. Samples were then spun down to collect tail hairs and supernatant was transferred to a fresh tube. Equal amount of iso-propanol was added to the samples and mixed properly. Samples were then centrifuged at 12000 rpm for 10 min at 4 °C and then washed twice with 70% ethanol. Samples were then air-dried and dissolved in DEPC water at 60 °C, quantified and stored at 4° C. For genotyping by PCR, 50-100 ng of DNA was taken and added with the optimized concentration of PCR mix and primers and

then run in a PCR machine. Amplified samples were then visualized by agarose gel electrophoresis.

3.2.15 Statistical analysis

Data are depicted as means ± S.E.M. Unpaired Student's t-tests were used to detect statistically significant differences between groups. P values lower than 0.05 were considered statistically significant. Statistical analyses and graphical presentations were computed with Graph Pad Prism, version 7.03 (GraphPad Software,USA).

4 RESULTS

4.1 FcεRIγ is extensively expressed on NK cells intracellularly

Foremost, to determine whether FcεRIγ is critically involved in the regulatory function of NK cells, we examined the expression of FcεRIγ by NK cells retrieved from Wild type (WT) and *Fcer1g*$^{-/-}$ mice. We found that FcεRIγ- intact NK cells demonstrated higher expression of intracellular FcεRIγ but negligible surface expression of FcεRIγ, whereas it is absent in *Fcer1g*$^{-/-}$ mice (Fig 4.1).

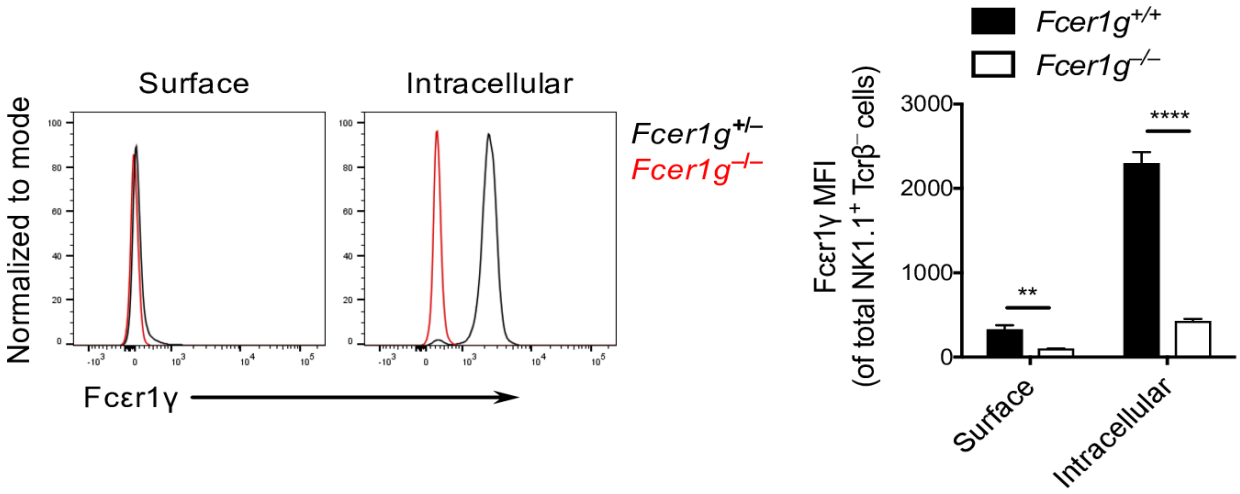

Fig 4.1: FcεRIγ is extensively expressed on NK cells intracellularly.
Surface and intracellular FcεRIγ expression by natural killer (NK) cells from spleens of *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice that had been infected intravenously (i.v.) with 2×10^4 plaque-forming units (PFU) of the lymphocytic choriomeningitis virus strain Docile (LCMV-Docile). Cells were analyzed 48 hours after infection *(n=4)*. Data are shown as mean ± SEM. Significant differences between the two groups were detected by unpaired two-tailed *t*-tests and are indicated as follows: ** $p<0.01$; **** $p<0.0001$. (Duhan *et al.*, 2019)

4.2 FcεRIγ has a prime role in NK cell-dependent downregulation of antiviral CD8$^+$ T cells

It is well established that, NK cells has a potential to shape the adaptive immune responses represented by antiviral CD8$^+$ T cell response in the course of LCMV infection (Lang *et al.*, 2012, Xu *et al.*, 2014, Crouse *et al.*, 2014). The molecular mechanisms employed by NK cells to calibrate this regulatory function remain to be fully defined. Further, IFN-I signalling protects CD8$^+$ T cells from NK cell–mediated cytotoxicity as demonstrated by high

susceptibility of interferon-α/β receptor deficient (*Ifnar*$^{-/-}$) CD8^{+} T cells to NK cell-mediated killing (Crouse *et al.*, 2014, Xu *et al.*, 2014).

To address the potential of FcRγ in the NK cell regulation of CD8^{+} T cell activity including their role in IFN-I mediated protection of T cells response, we adoptively transferred either WT P14 cells (WT P14) or P14 cells that lack the receptor for IFN-I (*Ifnar*$^{-/-}$ × P14) into *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice and then infected the mice with LCMV-Docile strain (Fig 4.2A). After 6 and 9 days of infection, we found that the number and frequency of WT P14 cells were markedly increased in *Fcer1g*$^{-/-}$ compared to *Fcer1g*$^{+/-}$ mice (Fig 4.2B, left panel). In parallel, the number of *Ifnar*$^{-/-}$ P14 cells, which show high susceptibility to NK cells killing, was vanished in *Fcer1g*$^{+/-}$ mice and partially rescued in *Fcer1g*$^{-/-}$ mice as compared to that of WT P14 transfer after infection with LCMV-Docile (Fig 4.2B, right panel). To extend our findings regarding NK cell–mediated killing of antiviral CD8^{+} T cells via FcεRIγ, we depleted NK cells in *Fcer1g*$^{-/-}$ mice and its littermates and analyzed the expansion of *Ifnar*$^{-/-}$ P14 cells after LCMV infection. In agreement, *Fcer1g*$^{-/-}$ mice showed higher CD8^{+} T cell expansion similar to that found in NK cell–depleted WT or *Fcer1g*$^{-/-}$ mice (Fig 4.2C). Together, these findings revealed that FcRγ has a cardinal role in NK cell-dependent regulation of antiviral CD8^{+} T cells during LCMV infection.

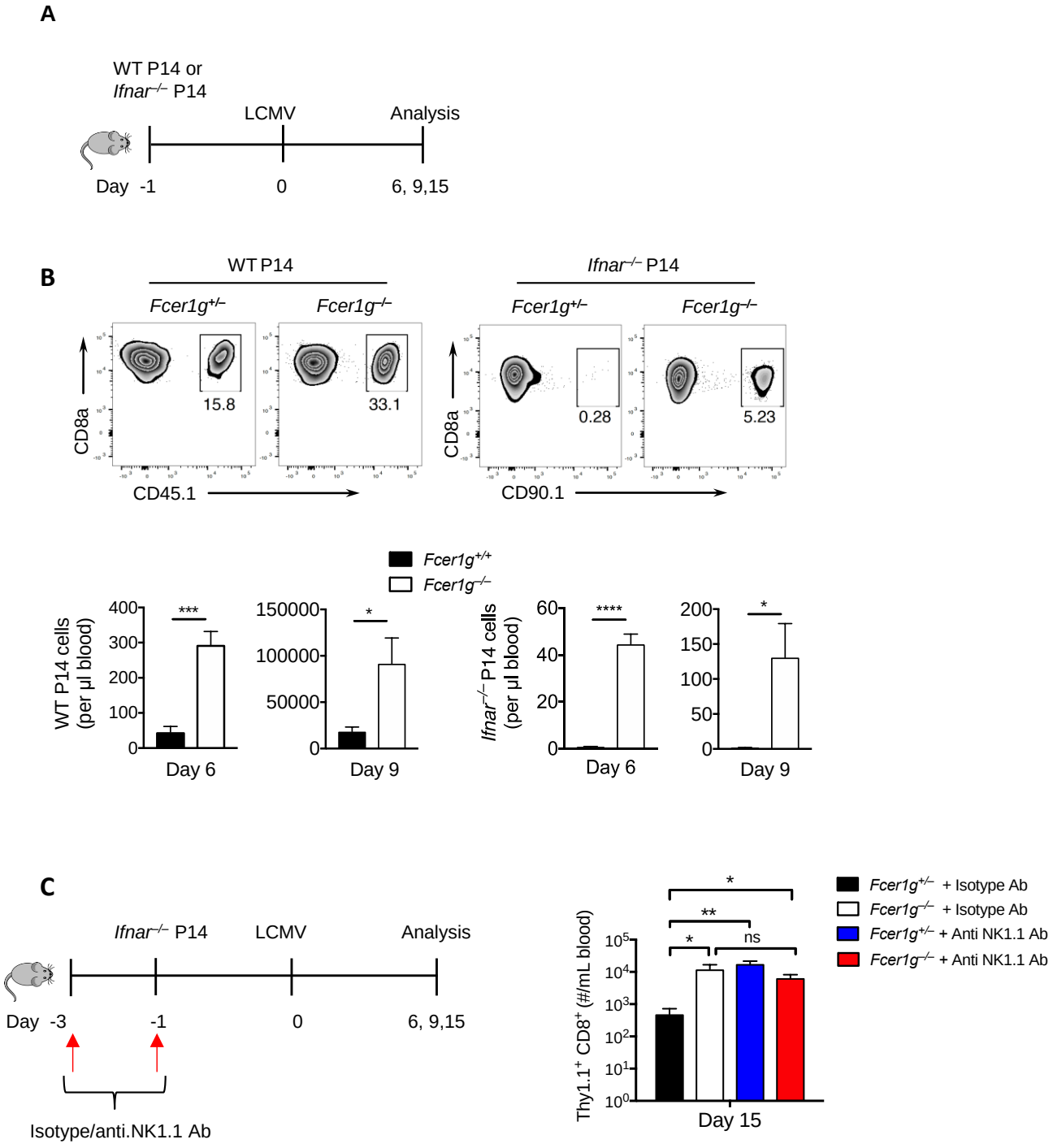

Fig 4.2: FcεRIγ has a prime role in NK cell-mediated downregulation of antiviral CD8+ T cells. (A) Schematic of the experimental setup. **(B)** Splenocytes (10^4) from WT P14 or *Ifnar*$^{-/-}$ x P14 mice were adoptively transferred into *Fcer1g*$^{+/-}$ or *Fcer1g*$^{-/-}$ mice one day earlier, then the mice were i.v. infected with 2×10^4 PFU of LCMV-Docile. In the upper panel, shown are representative histograms for the frequencies of WT P14 or *Ifnar*$^{-/-}$ P14 cells at day 6 post-infection. In the lower panel, the bar graph represents total number of transferred WT P14 or *Ifnar*$^{-/-}$ P14 cells in the blood at the indicated days after infection *(n=4).*

(C) 10^4 splenocytes from P14× *Ifnar*$^{-/-}$ mice were transferred into *Fcer1g*$^{+/-}$ or *Fcer1g*$^{-/-}$ mice that had been treated with isotype antibody or anti NK1.1 antibody at day 3 and 1 before i.v infection with 2×10^4 PFU of LCMV-Docile. The graph shows the total number of transferred P14 cells in blood at day 15 post-infection *(n=3-4).* Data are shown as mean ± SEM. Significant differences between the two groups were detected by unpaired two-tailed *t*-tests and are indicated as follows: ns, not significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. (Duhan *et al.*, 2019)

4.3 FcεRIγ deficiency does not alter the NK cell responses upon LCMV infection

To investigate whether NK cells lacking FcεRIγ are different from FcεRIγ sufficient NK cells in a steady state and during LCMV infection, we compared NK cells from *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice. In naïve mice and in mice infected with LCMV docile strain for 2 days, NK cells numbers and frequency were comparable (Fig 4.3A). Moreover, the percentage of IFN-γ, granzyme B, CD107a and perforin producing NK cells were similar in absence and presence of FcRγ during early LCMV infection (Fig 4.3B). Consistently, when we co-cultured activated NK cells from FcεRIγ -sufficient and -deficient animals with YAC-1 cells, which are vulnerable to NK cell mediated lysis, no differences of cytotoxicity were observed between *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice (Fig 4.3C). NK cell maturation can be dissected based on different expression of surface markers in the following order of their development: CD11b^{lo} CD27^{lo}, CD27^{hi} CD11b^{lo}, CD27^{hi} CD11^{hi} and CD27^{lo} CD11b^{hi} and they are related to the acquisition of NK cells effector function (Chiossone *et al.*, 2009). To study the impact of FcεRIγ missing on NK cells maturation, NK cells maturation subsets were examined after LCMV docile strain infection and we noticed that mature NK cells phenotype (CD27^{lo} CD11b^{hi}) were comparable between the two infected groups suggesting that NK cell maturation is not driven by FcεRIγ (Fig 4.3D). Collectively, the quantity and quality of NK cells were equivocal in *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice upon chronic LCMV infection

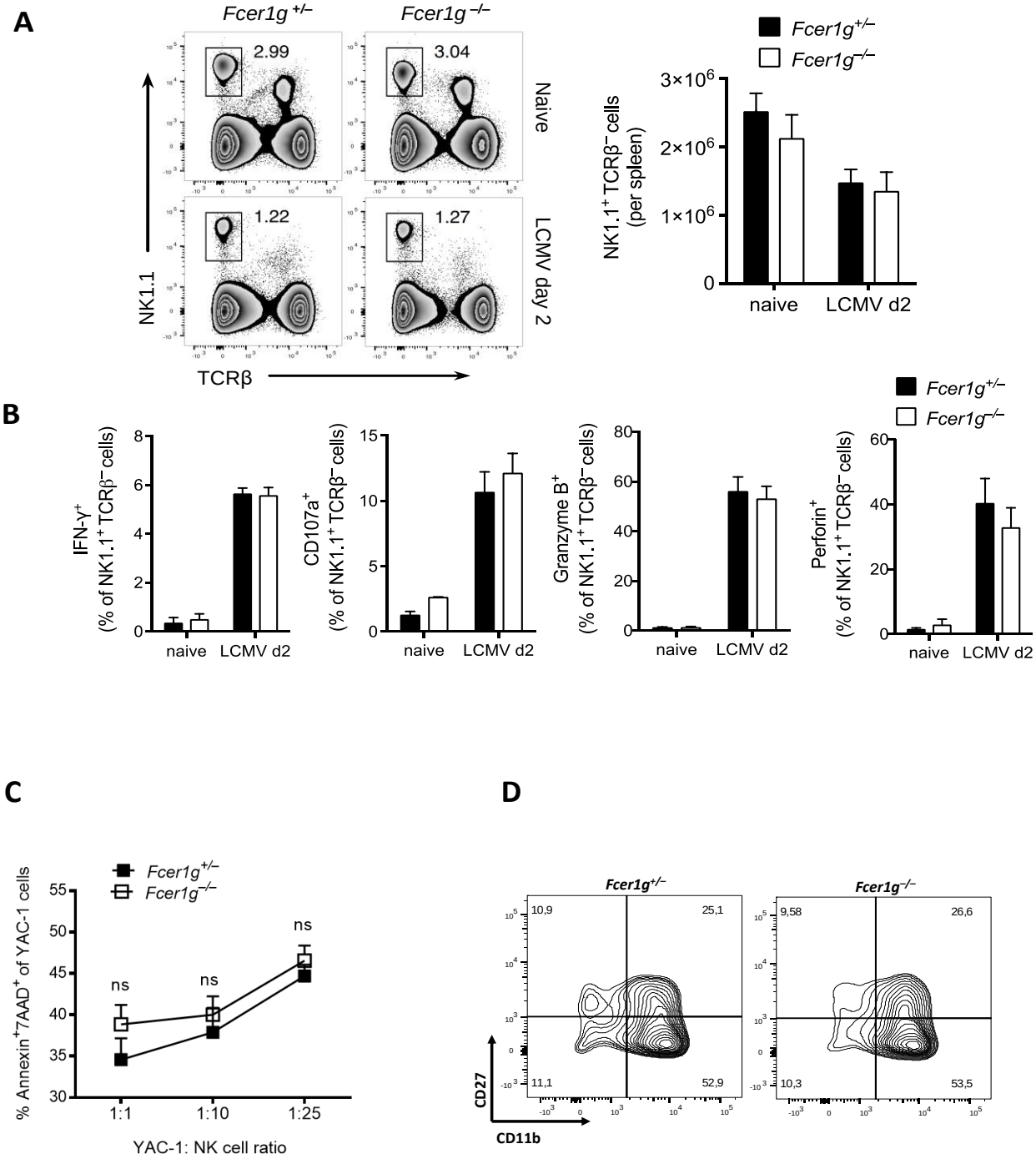

Fig 4.3: FcεRIγ deficiency does not alter the NK cell responses upon LCMV infection. *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice were left untreated or were infected i.v. with 2 x 10^4 PFU of LCMV-Docile. Mice were put to death on day 2 (d2) after infection and NK cells in the spleen were analyzed for various markers by flow cytometry. **(A)** Representative fluorescence-activated cell sorting (FACS) plots for the frequencies of NK cells (left panel). The bar graph in right panel shows total number of NK cells in naïve and LCMV-Docile

infected mice *(n=4)*. **(B)** Frequency of various markers in intracellularly stained NK cells from naïve and LCMV infected mice *(n=3-5)*. Data are pooled from two independent experiments. **(C)** Negatively sorted splenic NK cells harvested from *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ infected with LCMV- Docile for 48 hours. 20.000 YAC-1 cells were co-cultured with purified NK cells at the indicated ratios of NK to target cells for 5h followed by cytometric analysis. The percentage of apoptotic Annexin V and 7-AAD double positive YAC-1 cells is shown *(n=4)*. **(D)** *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice were infected intravenously with 2 × 10^4 plaque-forming units (PFU) of the Docile strain of lymphocytic choriomeningitis virus (LCMV) and were euthanized after 36 hours with *(n=4)*. The left contour graph depicts NK cells maturation subsets from splenic *Fcer1g*$^{+/-}$ murine model, and the right contour represents the one from *Fcer1g*$^{-/-}$ mouse. Data are shown as mean ± SEM. Significant differences between the two groups were detected by unpaired two-tailed t-tests and are indicated as follows: ns, not significant. (Duhan *et al.*, 2019)

4.4 The killing function of FcεRIγ-compromised NK cells is moderately impaired

To test the impact of FcRγ on the killing potential of NK cells upon LCMV infection. For this, we measured TNF-related apoptosis-inducing ligand (TRAIL) expressing NK cells, which have a key role in antiviral defense in *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice (Wang and El-Deiry, 2003), and we found that TRAIL expression was downregulated in mice the are devoid of FcεRIγ (Fig 4.4A). Similarly, FcεRIγ sufficient–mice demonstrated upregulated expression of protein kinase C theta (PKC-θ), which is needed for the sustainability of NK cell functions (Tassi *et al.*, 2008), compared to FcεRIγ-deficient mice (Fig 4.4B). Altogether, the reduced killing ability in *Fcer1g*$^{-/-}$ NK cells could be explained by reduced expression of TRAIL and PKC-θ.

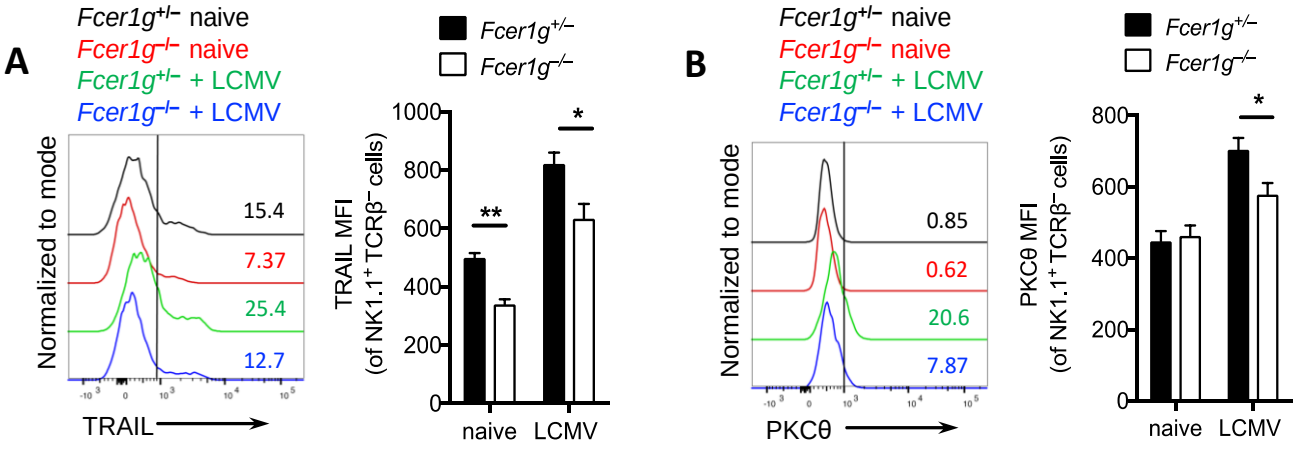

Fig 4.4: The killing function of FcεRIγ-compromised NK cells is moderately impaired. *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice were left untreated or were infected i.v. with 2 x 10^4 PFU of LCMV-Docile. Mice were put to death on day 2 (d2) after infection and NK cells in the

spleen were analyzed for various markers by flow cytometry. **(A)** Surface expression of TRAIL on splenic NK cells from naïve and LCMV-infected mice *(n=3-4)*. **(B)** Intracellular staining of PKC-θ on splenic NK cells from naïve and LCMV-infected mice *(n=3-4)*. Data are shown as mean ± SEM. Significant differences between the two groups were detected by unpaired two-tailed *t*-tests and are indicated as follows: ns, not significant; * $p<0.05$; ** $p<0.01$. (Duhan *et al.*, 2019)

4.5 FcεRIγ is prominent for NCR1 expression

To investigate whether absence of FcεRIγ could affect the inducible and constitutive expression of NK cell activation markers, we checked the expression of NK cell activation markers that are specific or nonspecific to NK cells or that are accompanied or not accompanied to FcεRIγ. We noticed the expression of NKG2D, Sca-1, Ly49H, CD69, CD27, and KLRG1 on splenic NK cells of *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ murine models were comparable. Intriguingly, we found that NCR1 was not phenotypically expressed on *Fcer1g*$^{-/-}$ NK cells, but on WT NK cells, suggesting that FcεRIγ is a unique component of NCR1 (Fig 4.5).

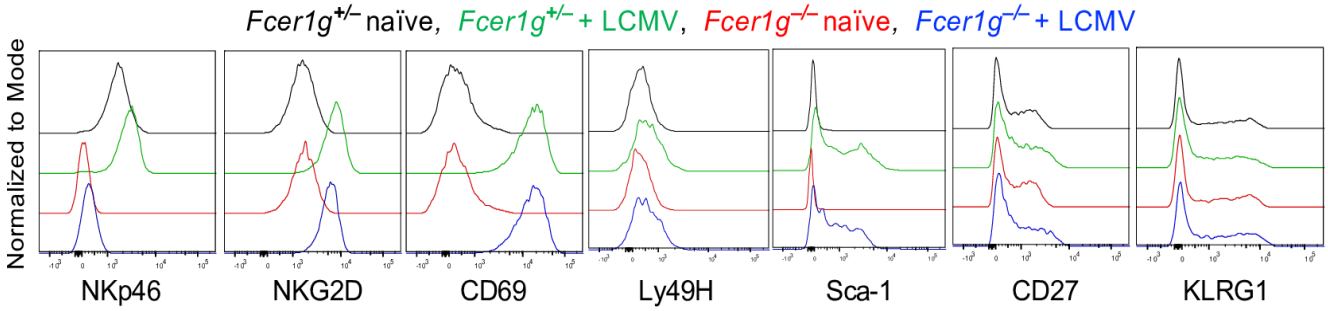

Fig 4.5: FcRγ is prominent for NCR1 expression. *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice were left untreated or were infected i.v. with 2 x 10^4 PFU of LCMV-Docile. Mice were put to death on day 2 (d2) after infection and NK cells in the spleen were analyzed for various markers by flow cytometry. Representative histograms for various cell surface markers on NK cells from naïve and LCMV-infected mice *(n=3-4)*. (Duhan *et al.*, 2019)

4.6 Immunoglobulin and Interferon-α/β receptor are dispensable for NKp46 expression

Next, we assess the role of IFN-I on NCR1 sufficiency. Therefore, we examined the expression of NCR1 on the WT and *Ifnar*$^{-/-}$ - derived NK cells, and found that the absence of interferon-α/β receptor on CD8 $^{+}$ T cells in *Ifnar*$^{-/-}$ mice has nothing to do with NCR1 expression on their NK cells indicating the independent intrinsic effect of NCR1 on *Ifnar*$^{-/-}$

T cells (Fig 4.6A). As aforementioned, Fc receptor is a major complementary for the immunoglobulin-Fc binding and Fc receptor is a unique constituent of NCR1 receptor. To find out if the missing of immunoglobulins can influence the NCR1 expression. We tested the expression of NK cell activating receptors on splenic NK cells isolated from *Jh*$^{-/-}$ mice which are devoid of B cells and hence all serum immunoglobulins, and we noticed the NKp46 was normally expressed on the splenic NK cells harvested from *Jh*$^{-/-}$ mice, which indicate the immunoglobulin- independent expression of NKp46. (Fig 4.6B).

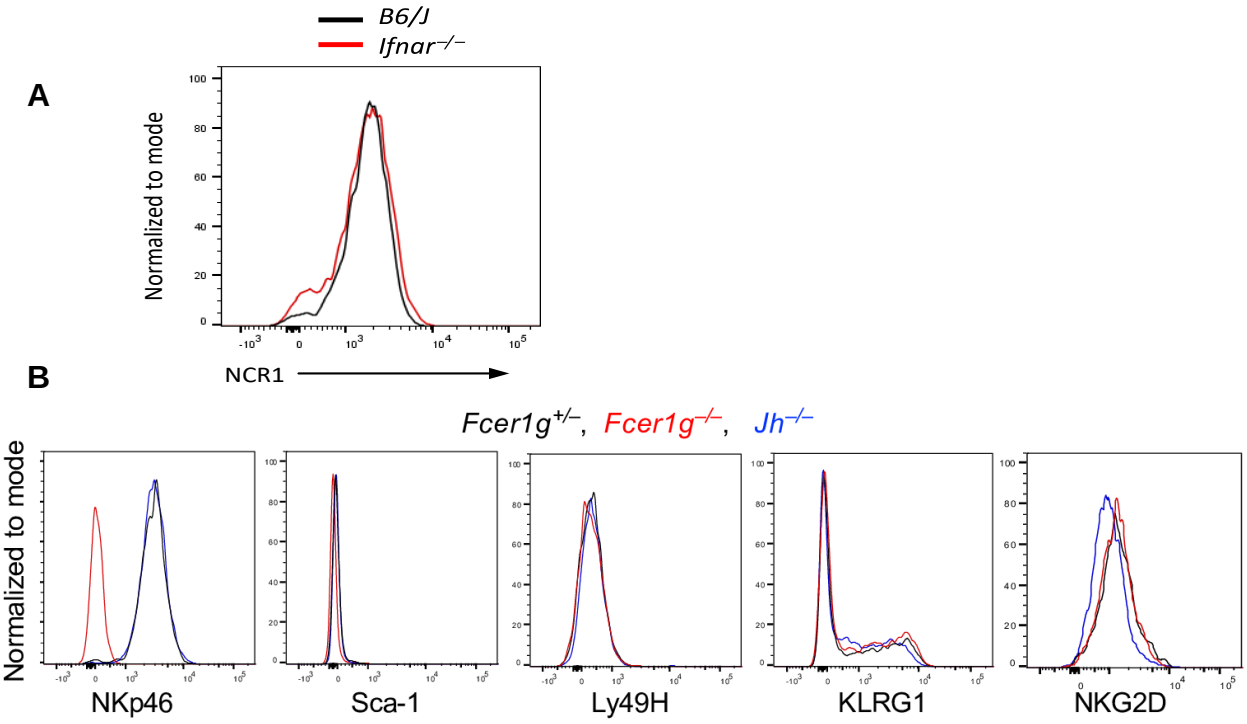

Fig 4.6: Immunoglobulin and Interferon-α/β receptor are dispensable for NKp46 expression. (A) Representative histogram for NCR1 expression on naïve splenic NK cells from B6/J and *Ifnar* $^{-/-}$ mice with (*n=3*). **(B)** Surface analysis of various markers on splenic NK cells of naïve *Fcer1g*$^{+/-}$, *Fcer1g*$^{-/-}$, and *Jh*$^{-/-}$ mice *(n=4)*. Shown histogram is a representative of three experiments. (Duhan *et al.*, 2019)

4.7 FcεRIγ is dispensable for NCR1 expression at mRNA level

To embark further on the underlying mechanism for the effect of FcεRIγ signaling on the NCR1 expression and to gain further insights into the stage of NCR1 regulation by FcεRIγ, here we tested the expression of FcεRIγ and NCR1 at mRNA level in sorted splenic NK cells cultured in presence of murine IL-2 harvested from WT and *Fcer1g*$^{-/-}$ animals. Unexpectedly, we found normal expression of NCR1 mRNA level in NK cells isolated from

Fcer1g$^{-/-}$ animal (Fig 4.7A), which hint that NCR1 undergo normal transcription process in FcεRIγ-deficient mice and the FcεRIγ does not regulate the NCR1 transcription or the stability of *NCR1* mRNA. A possible explanation for the expression of NCR1 mRNA in *Fcer1g*$^{-/-}$mice is that the NCR1 could internalize after its formation in the post-translation stage. To that end, we performed intracellular staining of NCR1 in splenic NK cells of WT and *Fcer1g*$^{-/-}$ mice, and we found no cytosolic NCR1 expression on *Fcer1g*$^{-/-}$ NK cells (Fig 4.7B), which exclude the intracellular sequestration/internalization of NCR1 protein.

Next, we questioned if the deficiency of FcεRIγ influence the expression of CD3 ζ, a unique compartment of NCR1 along with FcεRIγ. To answer this question, we measured the expression of CD3 ζ at mRNA and protein levels and we observed marginal upregulation of CD3 ζ at protein level on FcεRIγ-deficient NK cells compared to FcεRIγ-competent NK cells (Fig 4.7C).

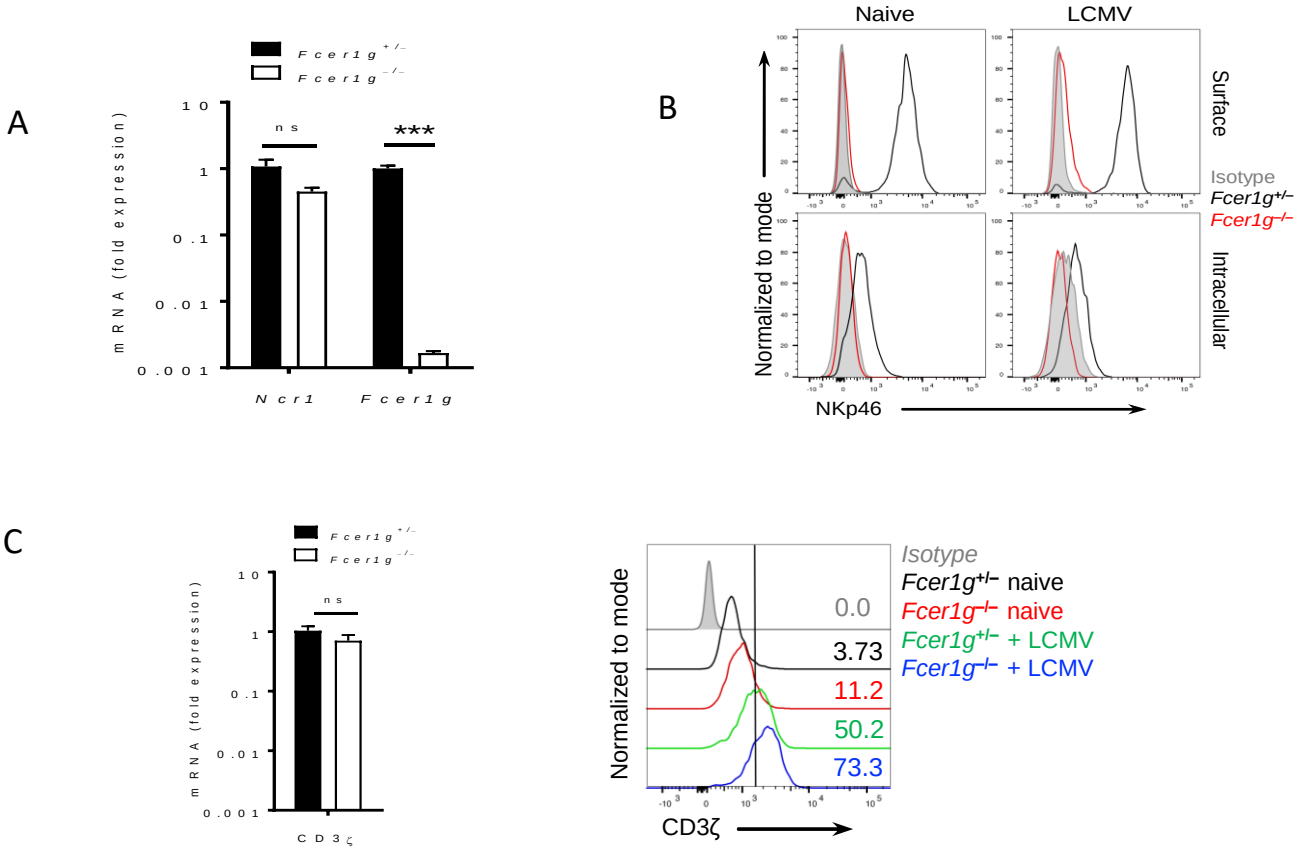

Fig 4.7: FcεRIγ is dispensable for NCR1 expression at mRNA level. (A) Bar graph showing the mRNA expression of NCR1 and FcεRIγ, as determined by RT-PCR from purified NK cells isolated from naïve spleens of *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice *(n=3).* **(B)** Histogram depicting surface and intracellular staining of NKp46 on splenic NK cells from naïve or infected *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice with 2 x 10^4 PFU of LCMV-Docile for 36h

(n=4). The histograms are representative of two independent experiments. **(C)** On the left panel, bar graph shows the mRNA expression of NCR1 and FcεRIγ, as determined by RT-PCR from purified NK cells isolated from naïve spleens of *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice *(n=3).* On the right panel, intracellular expression of CD3ζ on splenic NK cells from naïve or i.v infected *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice with 2 x 10^4 PFU of LCMV-Docile for 36 hours *(n=3-4).* Data are shown as mean ± SEM. Significant differences between the two groups were detected by unpaired two-tailed *t*-tests and are indicated as follows: ns, not significant; ** p<0. 01; *** p<0.001. (Duhan *et al.*, 2019)

4.8 NCR1 expression is stabilized by FcεRIγ

Next, we hypothesized that the NCR1 could undergo proteasomal degradation after its formation and accordingly its deficiency on NK cells of *Fcer1g*$^{-/-}$ mice. To that aim, we treated the cultured NK cells purified from *Fcer1g*$^{-/-}$ mice and WT with the proteasome inhibitor MG-132, and we observed restored expression of NKp46 in splenic NK cells derived from *Fcer1g*$^{-/-}$ mice in a similar fashion to NCR1 expression in WT (untreated) (Fig 4.8). These observations imply that NCR1 protein is stabilized by FcεRIγ and thereby prevent it from proteasomal degradation.

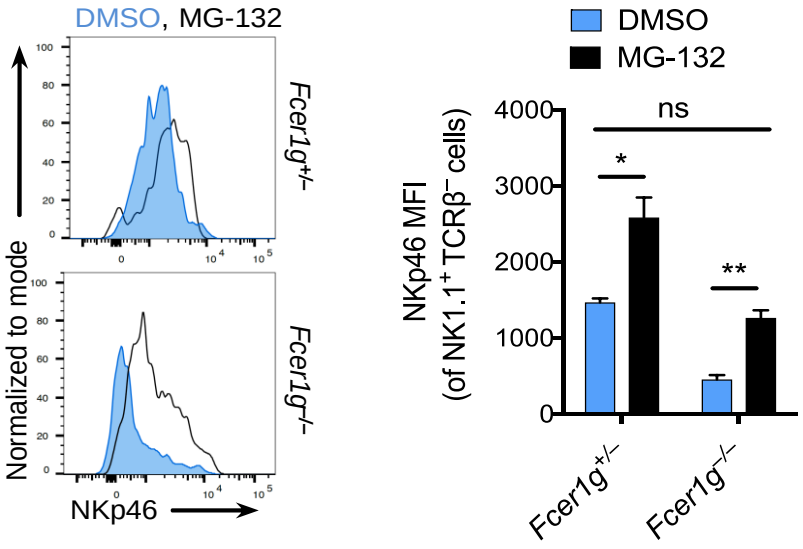

Fig 4.8: NCR1 expression is stabilized by FcεRIγ. Representative histogram for surface NKp46 expression on splenic NK cells from *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice treated *ex-vivo* with 20µg/ml MG-132 for 48 hours as indicated *(n=3) (left panel).* In the right panel, the shown is median fluorescence intensity (MFI) for the same experiment *(n=3).* Data are shown as mean ± SEM. Significant differences between the two groups were detected by unpaired two-tailed *t*-tests and are indicated as follows: ns, not significant; * p<0.05; ** p<0. 01. (Duhan *et al.*, 2019)

4.9 NK cell-intrinsic FcεRIγ deficiency is crucial to sustain a potent antiviral CD8$^+$ T cells during chronic LCMV infection

A substantial body of studies demonstrated that NK cells limit the virus-specific CD8$^+$ T cells. However, how exactly NK cells dampen CD8$^+$ T cells in the course of LCMV infection needs further investigations. Here, we sought to address if the virus-specific CD8$^+$ T cells are regulated by NK cells in an FcεRIγ-dependent manner. For this, we challenged adult *Fcer1g*$^{+/+}$ and *Fcer1g*$^{-/-}$ mice with LCMV-Docile and then analyzed CD8$^+$ T cell responses. The blood of *Fcer1g*$^{-/-}$ mice showed augmented polyclonal and LCMV-specific CD8$^+$ T cell responses compared to *Fcer1g*$^{+/+}$ littermates in terms of frequencies and numbers (Fig 4.9A). In agreement with the blood data, the spleen and liver tissues of *Fcer1g*$^{-/-}$ mice exhibited enhanced percentages and numbers of polyclonal and antiviral CD8$^+$ T cells at days 8 after infection (Fig 4.9B & C). Interestingly, antiviral CD8$^+$ T cells in *Fcer1g*$^{-/-}$ mice are more activated and functional as mirrored by upregulation of KLRG1 and downregulation of PD1 expression (Fig 4.9D). Consistently, the functionality of CD8$^+$ T cells are enhanced in the spleen and liver of *Fcer1g*$^{-/-}$ mice as documented by increased frequencies and total numbers of IFN-γ– and TNF-α–producing CD8$^+$ T cells (Fig 4.9E). Thus, these data demonstrate that the lack of FcεRIγ is crucial to sustain a potent virus-specific CD8$^+$ T cells in the settings of chronic LCMV infection.

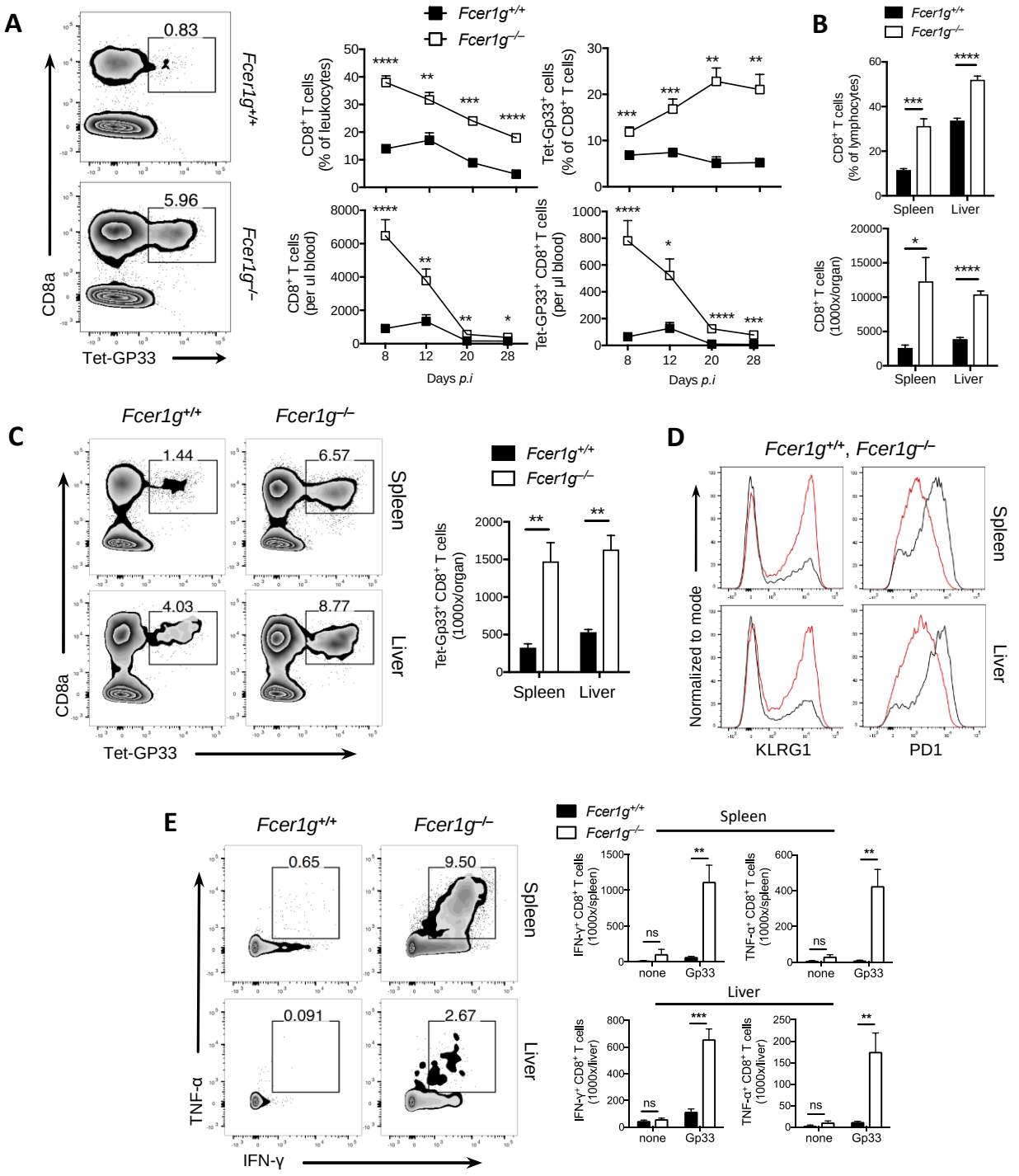

Fig 4.9: NK cell – intrinsic FcεRIγ deficiency is crucial to sustain a potent antiviral CD8$^+$ T cells during chronic LCMV infection.
Fcer1g$^{+/+}$ and *Fcer1g*$^{-/-}$ mice were infected i.v. with 2 × 10^4 PFU of LCMV-Docile and were bled at various time points or put to death on day 8 after infection. **(A)** The left representative FACS plot showing the frequency of glycoprotein (GP) 33-Tet$^+$ CD8$^+$ T cells of total

leukocytes in blood 8 days after infection. The right panel shows graphs of the kinetics for the frequency and number of $CD8^+$ T cells (middle; *n=3-12*) and virus-specific GP33-Tet^+ $CD8^+$ T cells in blood at the indicated time points (right; *n=3-12*). Data are pooled from 3 independent experiments. **(B)** Frequency and total number of $CD8^+$ T cells from spleen and liver on day 8 after infection *(n=4).* **(C)** Representative FACS plots and graphs showing the frequency and total number of GP33-Tet^+ $CD8^+$ T cells in spleens and livers on day 8 after infection *(n=4).* **(D)** Representative histogram showing the expression of PD1 and KLRG1 on GP33-Tet^+ $CD8^+$ T cells in spleens and livers on day 8 after infection *(n=4).* **(E)** FACS plots (left panel) and graphs (right panel) depict the percentage and total numbers of $CD8^+$ T cells producing interferon (IFN)-γ and tumor necrosis factor (TNF)-α in spleens and livers on day 8 after infection. The cells were stimulated *in-vitro* for 5 hours in the presence or absence of GP33 peptide *(n=4).* Data are shown as mean ± SEM. Significant differences between the two groups were detected by unpaired two-tailed t-tests and are indicated as follows:: ns, not significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. (Duhan *et al.*, 2019)

4.10 Virus-specific $CD8^+$ T cells are directly killed by NK cells in FcεRIγ-dependent manner

To address whether NK cells negatively regulate virus-specific $CD8^+$ T cells directly in FcεRIγ-dependent manner, we performed *in vivo* killer assay. To that aim, naïve P14 T cells were transferred into *Fcer1g*$^{-/-}$ mice followed by antigen challenge using LCMV-WE strain. After 5 days of in vivo activation, P14 were isolated and transferred into LCMV infected *Fcer1g*$^{+/-}$, *Fcer1g*$^{-/-}$ and NK cells-deficient naïve WT mice as a control (Fig 4.10A). Survival of transferred P14 cells were measured 4 hours later. The P14 seeded in FcεRIγ-deficient mice were dramatically increased compared to the FcεRIγ-sufficient mice and were dominated in the control group (Fig 4.10B). Collectively, NK cells-intrinsic FcεRIγ eliminates LCMV-specific $CD8^+$ T cells in a direct manner via NKp46.

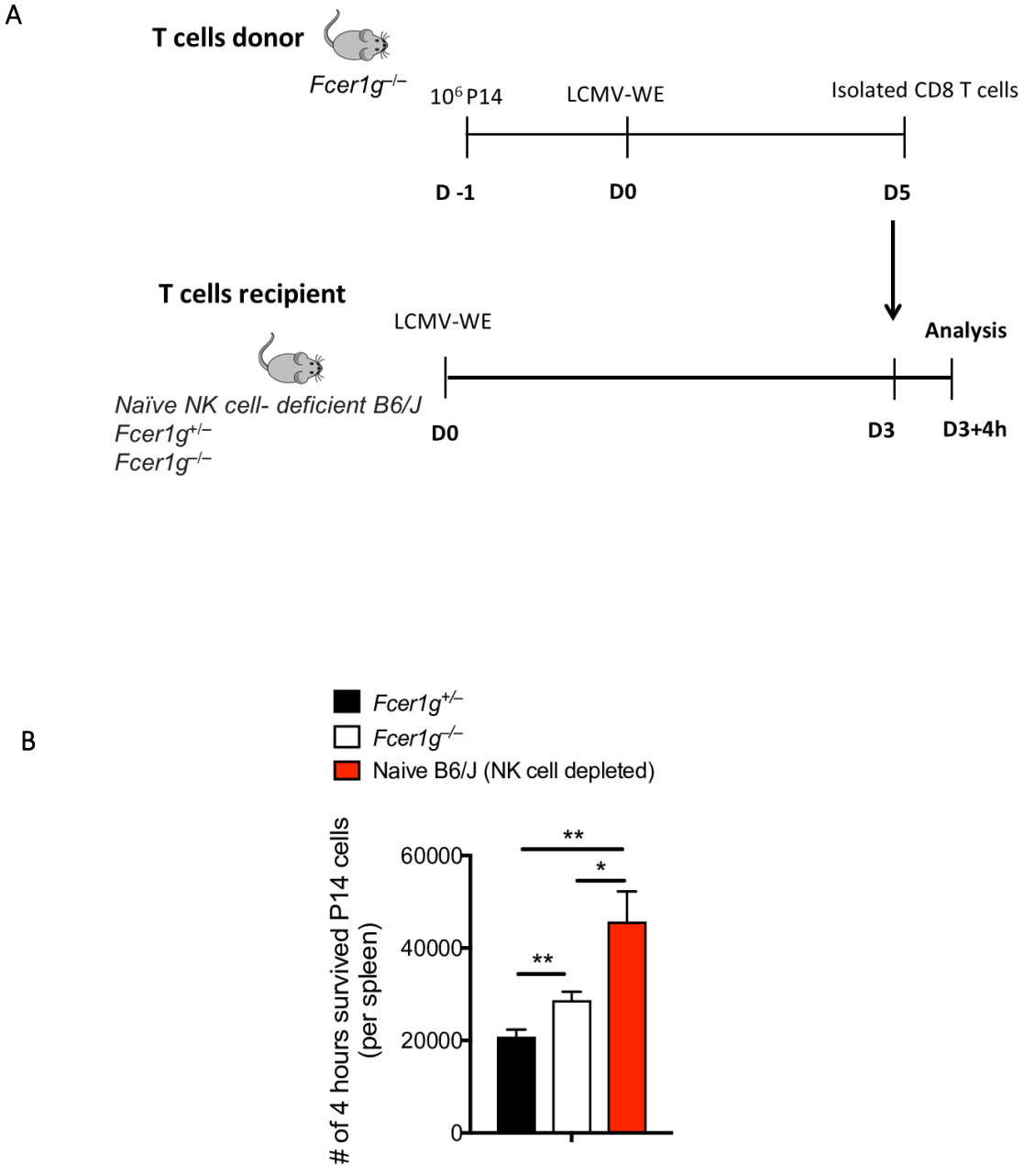

Fig 4.10: Virus-specific CD8⁺ T cells are directly killed in FcεRIγ-dependent manner. **(A)** Experiment setup for *in vivo* killer assay. The detailed protocol is described in materials and methods section (*in vivo* killer assay). **(B)** The bar graph represents the number of activated P14 cells after 4 hours of transfer in NK cells-depleted naïve C57BL6/J (B6/J) mice or *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$mice which were i.v infected with 200 PFU of LCMV-WE strain 3 days before the transfer. Data are pooled from 2 independent experiments *(n=6-7).* Data are shown as mean ± SEM. Significant differences between the two groups were detected by unpaired two-tailed *t*-tests and are indicated as follows: * $p<0.05$; ** $p<0.01$. (Duhan *et al.*, 2019)

4.11 NCR1 ligand is expressed on virus specific CD8$^+$ T cells, but is not influenced by FcεRIγ

To determine if the NCR1 ligand is expressed by virus specific CD8$^+$ T cells during the course of LCMV infection, P14 cells were adoptively transferred into WT and FcεRIγ -deficient mice, then infected with LCMV (2*10^4 P.F.U) then sacrificed 4 days later. We determined that NCR1 ligand is expressed on LCMV-specific CD8$^+$ T cells modestly suggesting that, LCMV specific CD8$^+$ T cells are targets for NK cells- mediated killing. Nevertheless, FcεRIγ sufficiency or deficiency exerts no effect on NCR1 ligand expression on the CD8$^+$ T cells (Fig 4.11).

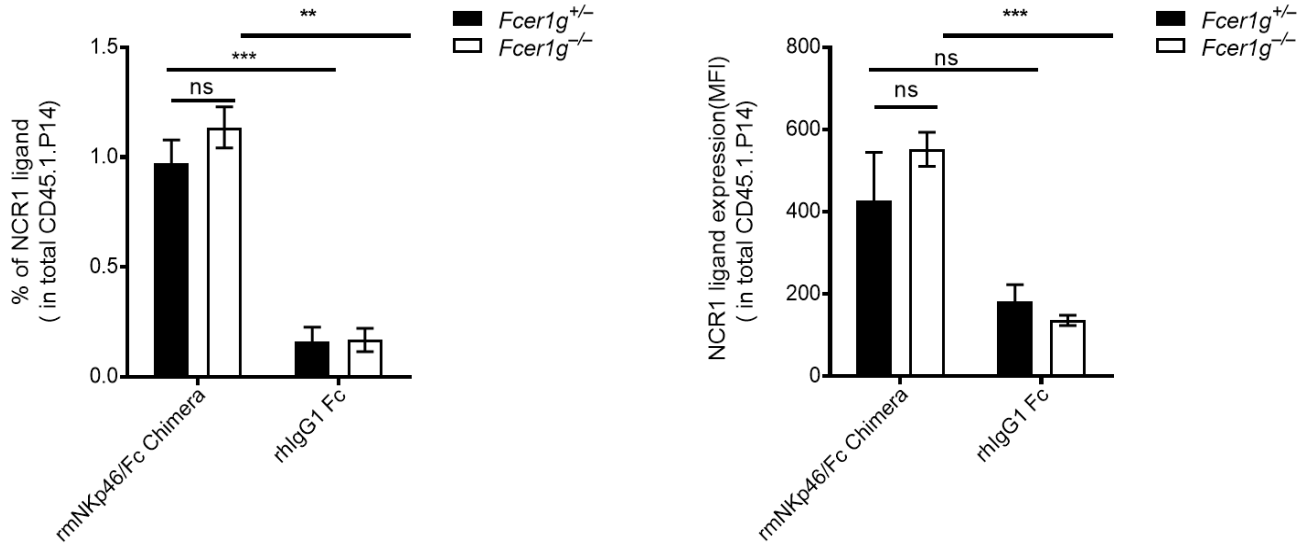

Figure 4.11: NCR1 ligand is expressed on virus specific CD8$^+$ T cells, but is not influenced by FcεRIγ. Splenocytes (2*10^6) were adoptively transferred from P14/CD45.1 one day before infection into two mice groups then infected with Docile strain of LCMV 2*10^4 P.F.U and euthanized after 4 days. In the left panel the Bar graph shows the NCR1 ligand expression frequency on LCMV-GP33 specific CD8$^+$ T (CD45.1$^+$ CD8$^+$ T cells) at day 4 after infection using murine NC1 fused with human Fc IgG1 along with negative control as described in the methodology with *(n=3)*. In the right panel, shown is the mean fluorescence intensity (MFI) for the same experiment. Significant differences between the two groups were detected by unpaired two-tailed *t*-tests and are indicated as follows: ns, not significant; ***p*<0.01; ****p*<0.001.

4.12 CD8$^+$ T cells have durable augmented response in deficiency of FcεRIγ during chronic LCMV infection

To get insight about the effect of FcεRIγ on CD8$^+$ T cell response in the course of long-term chronic LCMV infection, we tracked the LCMV-specific CD8$^+$ T cells and viral load in different organs until day 55. We demonstrated that the FcεRIγ has durable effect in curbing the CD8$^+$ T cells response as the latter is maintained *Fcer1g*$^{-/-}$ compared to the littermate (Fig. 4.12 A, B &C), nevertheless, the virus was resolved in the target organs in both murine models (Fig.4.12 D).

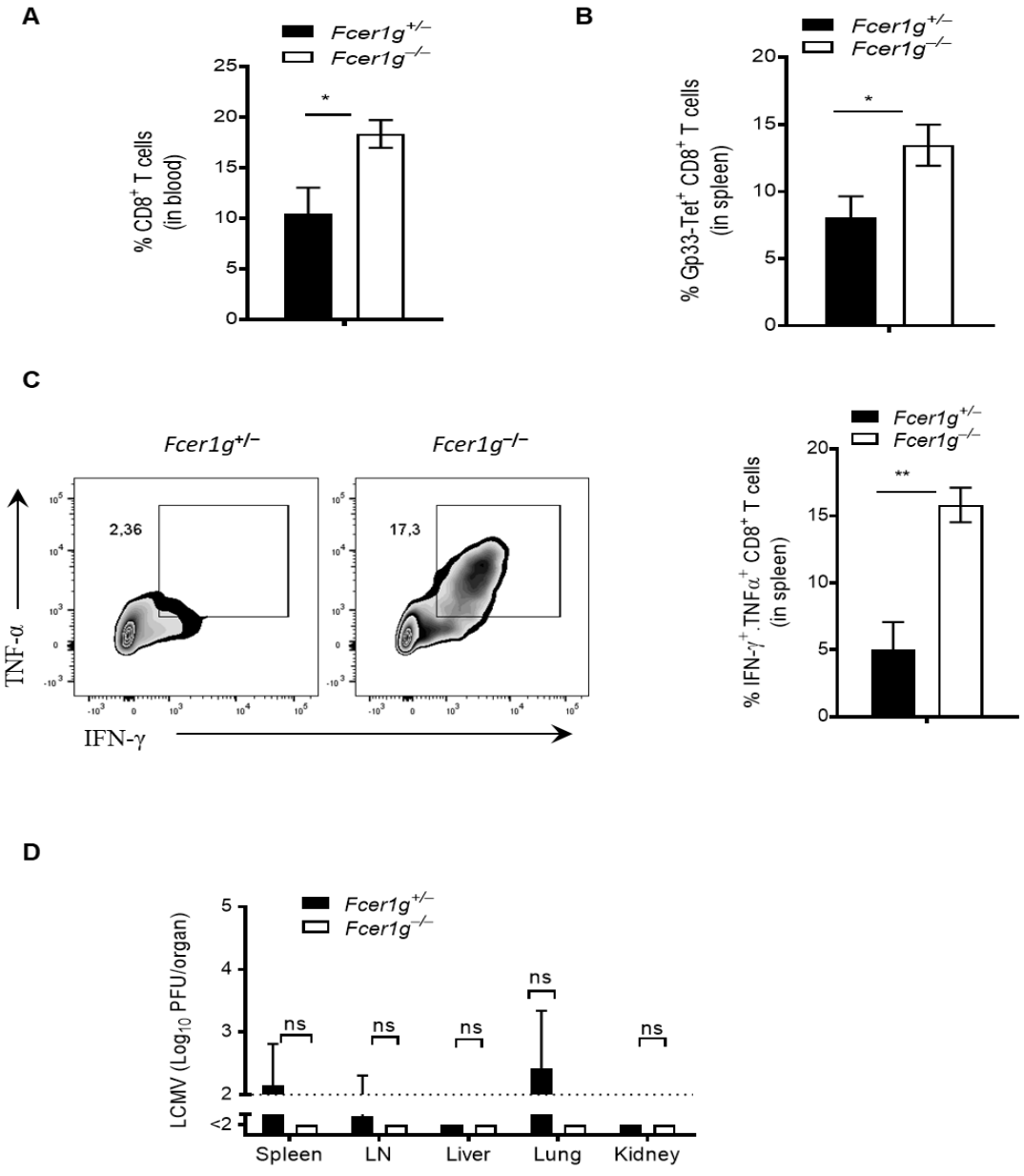

Figure 4.12: CD8$^+$ T cell has durable augmented response in deficiency of FcεRIγ during chronic LCMV infection. *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice were infected intravenously with 2×10^4 plaque-forming units (PFU) of the Docile strain of lymphocytic choriomeningitis virus (LCMV) and were bled and euthanized at day 55 with *(n=5).* **(A)** The bar graph depicts the frequency of CD8$^+$ T in blood at day 60 after infection. **(B)** The bar graph represents GP33-Tet+ CD8$^+$ T cell percentage in spleen. **(C)** In the left panel, shown is representative FACS plots depict the percentage (IFN) -γ and tumor necrosis factor (TNF)-α in spleens. In right panel, the bar graph depicts the frequency of (IFN) -γ and (TNF)-α produced by CD8$^+$ T cells **(D)** The bar graph shows the viral load from different lymphoid and non-lymphoid organs. Significant differences between the groups were detected with unpaired two-tailed t-tests and are indicated as follows: ns, not significant; *p<0.05; ** p<0.01. (Duhan *et al.*, 2019)

4.13 NK cell-intrinsic FcεRIγ aggravates viral elimination during chronic LCMV infection

To elucidate the impact of FcεRIγ deficiency on the viral control in the context of chronic viral infection. Here, we challenged WT and *Fcer1g*$^{-/-}$ mice with 2×10^4 plaque-forming units (PFU) of LCMV-Docile and assessed virus control. Consistent with robust CD8$^+$ T cell response in absence of FcεRIγ, LCMV titer was eradicated from the circulation and most of the organs in *Fcer1g*$^{-/-}$ mice within 12 days with confined reactivity in kidney (Fig 4.13 A–C). Dissimilarly, more viral titers were found in lymphoid and non-lymphoid organs harvested from FcεRIγ-sufficient mice (Fig 4.13A–C). Next, to assess the signs of immunopathology in *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice upon chronic LCMV infection, we measured the liver enzyme levels and body weight percentage as functional readouts of the hepatic damage. We noticed substantial immunopathology in *Fcer1g*$^{+/-}$ mice as indicated by higher liver enzymes level and less body weight, while *Fcer1g*$^{-/-}$ mice showed virtually no liver pathology and temporary weight loss during persistent viral infection due to fast clearance of virus by robust virus-specific CD8$^+$ T cell response (Fig 4.13D&E). More importantly, the liver pathology seen in the *Fcer1g*$^{+/-}$ mice was due to high virus replication in hepatocytes that are target for LCMV-specific CD8$^+$ T cell mediated killing and subsequently virus-induced immunopathology (Fig 4.13F). This demonstrates that absence of FcεRIγ leads to efficient viral eradication compared to FcεRIγ expressing WT mice.

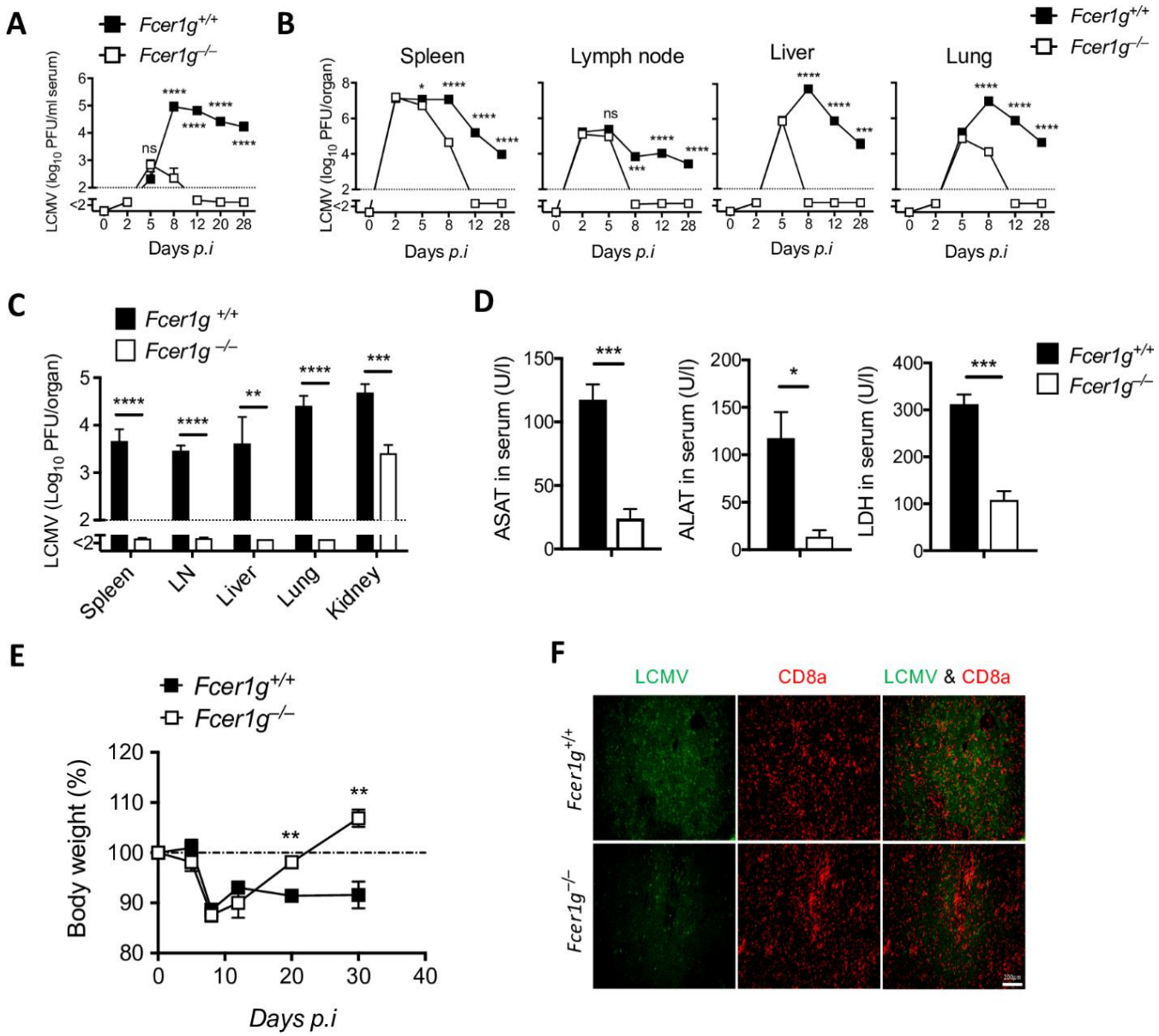

Fig 4.13: NK cell-intrinsic FcεRIγ aggravates viral elimination during chronic LCMV infection.

Several groups of *Fcer1g*$^{+/+}$ and *Fcer1g*$^{-/-}$ mice were infected i.v with 2 × 10^4 PFU of LCMV-Docile, were bled or killed at diverse time points, and were analyzed for certain variables. **(A)** Kinetics of viral titers in serum at the indicated time points after infection *(n=4-8).* Data are pooled from 3 independent experiments. **(B)** Kinetics of viral titers in various organs at the indicated time points after infection *(n=3-4).* **(C)** Viral titers in various organs on day 28 after infection *(n=7-8).* Data are pooled from 2 independent experiments. **(D)** Levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) measured in serum on day 12 after infection *(n=4).* **(E)** Percentage of body weight is shown at various days after infection *(n=5).* **(F)** Representative immunofluorescence for liver histological sections from *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice stained for LCMV nucleoprotein (green) and CD8$^+$ T cells (red) at day 12 after infection. One slide representative of 4 slides is shown. Scale bar, 200μm. Data are shown as mean ± SEM. Significant differences between the two groups were detected by unpaired two-

tailed *t*-tests and are indicated as follows: ns, not significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. (Duhan *et al.*, 2019)

4.14 NK cell depletion reveals the inherent role of FcεRIγ on CD8$^+$ T cells response and virus control

Next, we wondered whether the NK cells depletion in the *Fcer1g*$^{-/-}$ mice could affect antiviral CD8$^+$ T cells and viral control. To this end, we depleted NK cells from *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice and infected the mice with LCMV-Docile (Fig 4.14A). NK cells elimination in *Fcer1g*$^{+/-}$ mice rescued the abortive antiviral CD8$^+$ T cells in terms of magnitude and functionality of virus-specific CD8$^+$ T cells to the level found in *Fcer1g*$^{-/-}$ mice which were treated with isotype antibody (Fig 4.14B & C). In addition, CD8$^+$ T cells response was unchanged in FcεRIγ -deficient murine models upon NK cells depletion, due to the fact that NCR1, which has a cardinal role on negative shaping of anti-viral CD8$^+$ T cells, is already missing in *Fcer1g*$^{-/-}$ mice (Fig 4.14B & C). In line with antiviral CD8$^+$ T cells response findings upon NK cells ablation, virus control in NK cell–depleted WT mice was improved as *Fcer1g*$^{-/-}$ mice treated with anti-NK1.1 or isotype antibody in most lymphoid, non-lymphoid compartments as well as in the bloodstream (Fig 4.14 D & E). Altogether, these findings indicate the inherent role of NCR1- intrinsic FcεRIγ in negative regulation of virus-specific CD8$^+$ T cells in the setting of chronic infection.

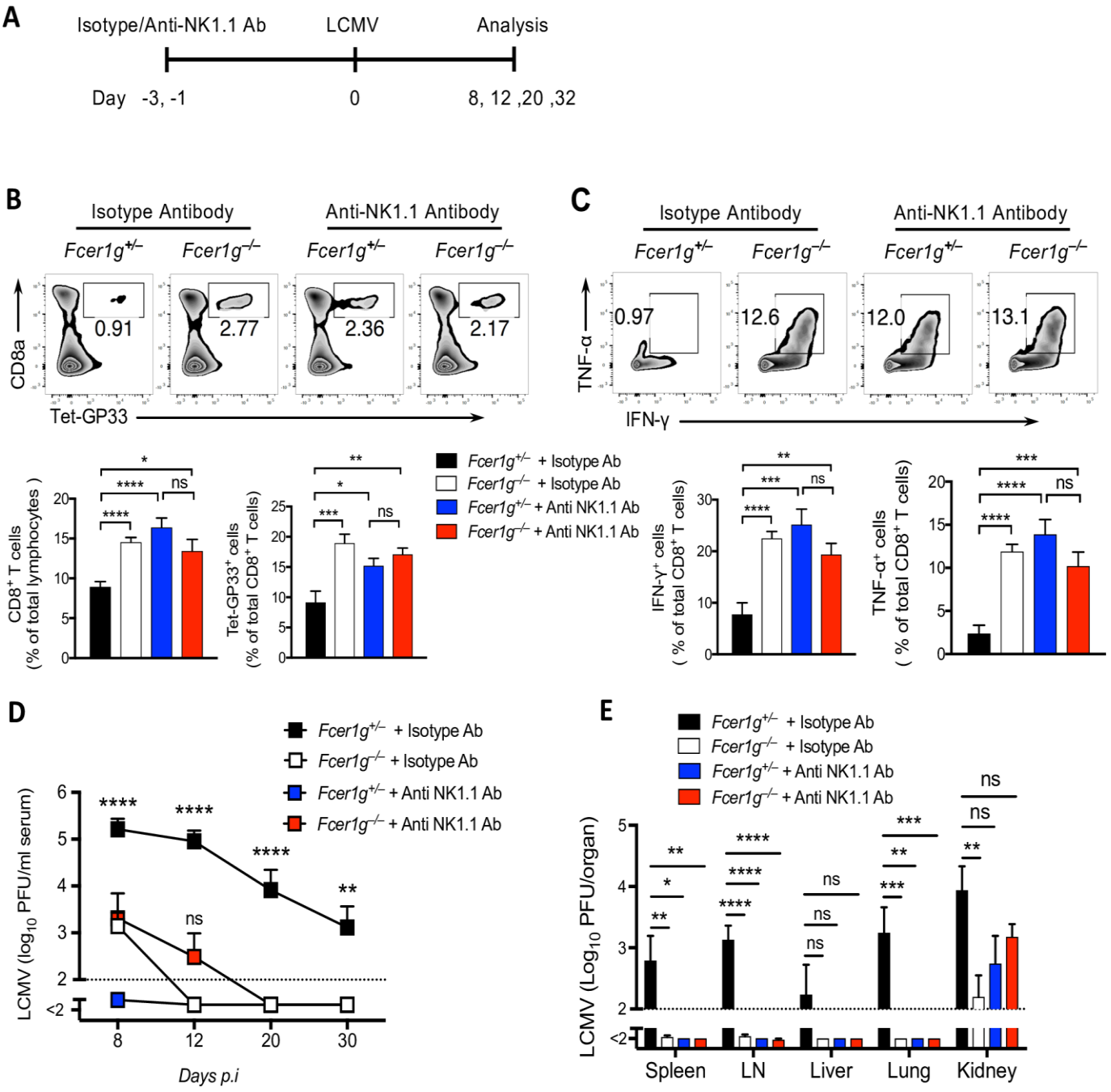

Fig 4.14: NK cell depletion reveals the inherent role of FcεRIγ on CD8⁺ T cells response and virus control.

(A) Schematic of experimental setup. *Fcer1g*$^{+/-}$ and *Fcer1g*$^{-/-}$ mice were injected intraperitoneally with 200ug of anti-NK1.1 or isotype antibody on day -3 and day -1 and were infected i.v with 2×10^4 PFU of LCMV-Docile at day 0. The mice were bled on days 8, 12, 20, and 32 after infection and were put to death on day 32 after infection. **(B)** The upper panel shows representative FACS plots for the frequency of glycoprotein (GP)33-Tet$^+$ CD8$^+$ T cells in the spleens on day 32 after infection. The lower panel shows graphs indicating the frequencies of CD8$^+$ T cells and GP33-Tet$^+$ CD8$^+$ T cells in murine spleens on day 32 after infection *(n=6-10)*. **(C)** The FACS plots (upper panel) and graphs (lower panel) show the percentages of CD8$^+$ T cells producing IFN-γ and TNF-α from splenocytes

on day 32 after infection. These cells were stimulated *in-vitro* for 5 hours in the presence of GP33 peptide (*n=6-10*). **(D)** Kinetics of viral titers in serum at indicated time points *(n=7-10).* **(E)** Viral titers from various organs on day 32 after infection *(n=7-10).* Data are pooled from two independent experiments (B-E). Data are shown as mean ± SEM. Significant differences between the groups were detected by unpaired two-tailed *t*-tests and are indicated as follows: ns, not significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. (Duhan *et al.*, 2019)

4.15 FcεRIγ is ubiquitously expressed on different immune cells

To analyze the cellular distribution of FcεRIγ adaptor molecule on different immune cells, we examined the expression of FcεRIγ on a variety of naïve innate and adaptive immune cells. We found out that, FcεRIγ is expressed intracellularly on Macrophages, cDCs, Granulocytes, Monocytes, NK cells and modestly on helper and cytotoxic T cells, indicating that FcεRIγ has diverse functions that is not restricted to NK cells only (Fig 4.15).

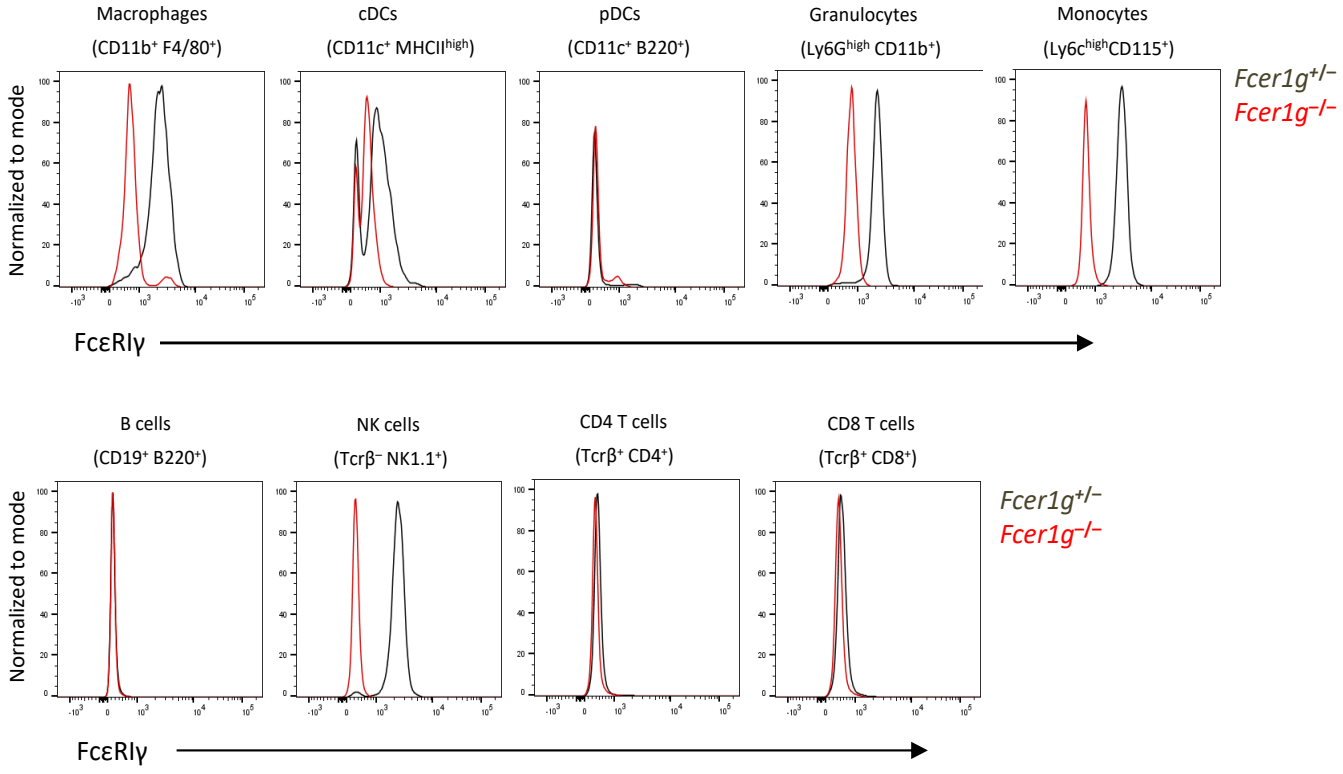

Figure 4.15: FcεRIγ is ubiquitously expressed on different immune cells.
Representative histogram for the expression of FcεRIγ on different naïve splenic innate and adaptive immune cells with *(n=4*). (Duhan *et al.*, 2019)

5 DISCUSSION

5.1 The impact of FcεRIγ on NK cell potential

FcRγ/FcεRIγ subunit is widely expressed on a variety of immune cells and plays a myriad of regulatory roles in the immune system because of their structural diversity. Apart from their indispensable role in specific binding to the Fc portion of antibody subsets, FcRγ/FcεRIγ manifests diverse biological functions upon binding to their putative ligands. Examples of such manifestations abound phagocytosis, presentation of antigens, mediation of antibody-dependent cellular cytotoxicity, anaphylactic reactions, and promotion of apoptosis of T cells and natural killer cells (Nimmerjahn and Ravetch, 2008). Besides their broad expression on different immune cells, we found that FcεRIγ is extensively expressed on NK cells intracellularly. However, FcRγ deficiency has no influence on the quantity of the NK cells at steady or activated status. Functionally, lack of FcRγ does not alter the effector functions of NK cells as documented by comparable frequencies of NK cells producing IFN-γ, granzyme B, and perforin in FcεRIγ-sufficient and FcεRIγ-deficient mice. NK cells employ different killing mechanisms to mount the peril, of these; exocytosis of granzymeB/perforin, TRAIL pathway as well as ADCC (Smyth *et al.*, 2002). FcRγ NK cells demonstrated reduced ability to express TRAIL, in line with recent study that found that NK cells that are devoid of NCR1, in which the FcRγ is a key subunit, lack surface expression of TRAIL (Sheppard *et al.*, 2018), which could work synergistically with lack of NCR1 to result in higher expansion of antiviral $CD8^+$ T cells. Likewise, analysis of PKCθ expression, a pillar component of downstream signalling of killer activating receptors (KARs) that induces the activation of NK cells, showed reduced expression in murine models that are devoid of FcεRIγ (Anel *et al.*, 2012). This finding suggests that KAR-induced activation is reduced in FcεRIγ-deficient NK cells, but this requires further study for clarification.

Further analysis of NK cells in FcεRIγ-sufficient and–deficient mice exhibited no difference in NK cells maturation, in consistent with other study which demonstrate equivocal NK cells maturation in *Ncr1*$^{gfr/gfr}$ and WT (Sheppard *et al.*, 2013). Activation of NK cells and their functions are regulated by both activating and inhibitory signals (Brandstadter and Yang, 2011). Assessing different NK cells specific activation markers upon LCMV infection showed no hyperactivity of splenic NK cells retrieved from *Fcer1g*$^{-/-}$ mice. Thus, NK cell development and activation is independent of FcRγ signaling. In parallel with our findings,

NK cells of NCR1$^{gfp/gfp}$ mice were found normal in terms of quality, quantity and development, despite the fact that NCR1$^{gfp/gfp}$ mice succumbed to influenza virus infections and had impaired efficiency in eradication of MHC class I–deficient RMAS tumor (Sheppard *et al.*, 2013, Gazit *et al.*, 2006, Pallmer *et al.*, 2019). In contrast, NK cells of *Ncr1*$^{Noé/Noé}$ and *NKp46*$^{icre/icre}$ mice were reported hyperactive due to point mutation in *Ncr1* gene. This point mutation resulted in resistance to MCMV and influenza virus infections (Narni-Mancinelli *et al.*, 2012).

5.2 FcεRIγ as a unique subunit in NCR1

A previous study in healthy individuals revealed a novel subset of human NK cells that are deficient in FcRγ with reduced expression in NKp46/NCR1 and exhibited poor reactivity toward tumor targets (Hwang *et al.*, 2012). Confirming the findings by Hwang *et al*, NK cells that are devoid of FcεRIγ were found to be negative for NCR1 expression. Thus, the absence of NCR1 in *Fcer1g*$^{-/-}$ mice was not due defect on NK cell differentiation or activation, as we observed comparable activation profile in the NK cells retrieved from WT and *Fcer1g*$^{-/-}$ mice. Nevertheless, the NCR1 deficiency was direct consequence of FcεRIγ absence. Moreover, low concentrations of NCR1 have been noted in several clinical settings, including acute myeloid leukemia, human immunodeficiency virus 1 infection and bare lymphocyte syndrome, type I; nevertheless, this downregulation NCR1 was not due to FcRγ insufficiency (Gazit *et al.*, 2006). Yet, the impact of FcRγ during chronic viral infection remains to be investigated. It is likely that, this complete lack of NKp46 in FcεRIγ-deficient NK cells would affect the natural killing activity induced by NKp46 signalling.

FcRγ is accompanied with NCR1 in transmembrane region. Moreover, the transmembrane part of FcRγ is disulfide bonded to CD3ζ (encoded by CD247 gene) and both of them contain ITAMs in cytoplasmic region which initiate signalling downstream to NKp46 ensuing in NK cell activation (Hudspeth *et al.*, 2013). In this study, we found marginal enhanced expression of CD3ζ in the absence of FcRγ compared to competent mice; this increased expression could be served as a compensatory mechanism to provide efficient downstream signalling to the NKp46/FcRγ/CD3ζ complex. A finding indicating that NKp46 expression is independent of CD3ζ. In sharp contrast, another study demonstrated that NKp46 expression is dependent on FcRγ and CD3ζ (Walzer *et al.*, 2007). In another study conducted

by Arase *et al*, they found that CD16 expression is upregulated on CD3ζ $^{-/-}$ mice. This finding could reveal a negative regulatory role of FcRγ on CD3ζ expression (Arase *et al.*, 2001).

No effect of immunoglobulins deficiency on the NCR1 expression was detected which implies that FcεRIγ-induced expression of NKp46 by NK cells was independent of the cross-linking of Fc receptors with antibodies. Type I interferons (IFNs) and different other cytokines are essential for natural killer (NK) cell homeostasis and function (Muller *et al.*, 2017), nevertheless, NCR1 expression was intact on IFNAR deficient mice-derived NK cells, indicating that the autonomous IFN-I signaling is dispensable for NCR1 expression. While FcεRIγ deficiency led to phenotypical lack of NCR1, it has no impact on NCR1 transcription as supported by normal expression of NCR1 in FcεRIγ -deficient mice at mRNA level. Because levels of the *Ncr1* transcripts were comparable in both mouse strains, we sequenced the DNA from WT and *Fcer1g*$^{-/-}$ and identified no altered nucleotides or point mutation in the signal peptide of NCR1 (Data not shown). The point mutation of signal peptide could affects its hydrophobicity that interfere with the binding of the mutant protein to the signal recognition particles, its insertion and translocation into ER and the protein maturation processes. Nevertheless, this is not the case here, as we did not detect point mutation in NCR1 gene such as the findings by Jang *et al*, who found no expression of NCR1 on CD45.1-derived NK cells due to point mutation in NCR1 transcripts (Jang *et al.*, 2018). A possible explanation of NCR1 expression at mRNA level, but not in the protein level in FcεRIγ- deficient mice is that, NCR1 could internalize after its formation in the post-translation stage. However, intracellular staining of NCR1 was negative in NK cells retrieved from WT and *Fcer1g*$^{-/-}$ mice. Rather than NCR1 internalization/sequestration, the protein degradation and instability could be the underlying mechanism for this phenomenon. Co-culturing the FcεRIγ -deficient NK cells with MG-132 enabled the restoration of NCR1 expression; implicating that NKp46 protein degradation could be rescued by inhibiting the proteasomal activity of NK cells, after *ex vivo* treatment with MG-132.

5.3 NK cell-intrinsic FcεRIγ link with antiviral CD8^{+} T cells

Effective T cell responses are crucial for the clearance of viral infection. One obstacle limiting the clearance of persistent infections is functional inactivation of antiviral T cells

During chronic viral infections, it was reported that CD8 T cells is downmodulated by different immune checkpoints such as; perforin, IL-10 and PD1 (Brooks *et al.*, 2006, Barber *et al.*, 2006, Matloubian *et al.*, 1999). A recent study showed that, innate cells have an important immunomodulatory role throughout chronic infection; a myeloid cell resembled myeloid-derived suppressor cells has a potential to suppress T cell proliferation during chronic viral infection (Norris *et al.*, 2013). Furthermore, tumor infiltrating T cells (TIL) were negatively regulated by NKp46 expressing innate lymphoid cells (Crome *et al.*, 2017). A substantial body of studies addressed the immunomodulatory function for NK cells as being rheostats for T cell responses during chronic viral infections (Waggoner *et al.*, 2011), but the underlying mechanisms have not fully confined. Here, we unravels that FcεRIγ is one major driver in T cell regulation by NK cells during chronic LCMV settings. Two recent studies demonstrated that IFNAR deficient $CD8^+$ T cells were preferentially recognized and eliminated by NK cells through NCR1 (Crouse *et al.*, 2014, Xu *et al.*, 2014).

Adoptive transfer of P14 cells that are IFNAR deficient led to dramatic reduction of virus-specific $CD8^+$ T cells in the WT mice, whereas, this reduction was less pronounced in *Fcer1g*$^{-/-}$mice. FcεRIγ -deficient NK cells are still able to kill the virus-specific CD8 T cells which are IFNAR deficient which are highly sensitive to NK cells mediated killing. Indeed, T cell immunity of *Ifnar*$^{-/-}$ × P14 T cells was restored by NK cell depletion in the WT mice while in the *Fcer1g*$^{-/-}$mice it renders the same expansion as in the untreated group. We found LCMV-specific $CD8^+$ T cells are more abundant and functional in chronically infected *Fcer1g*$^{-/-}$ mice compared to littermates. Virus-specific $CD8^+$ T cells produced higher levels of IFN-γ and TNF-α in FcεRIγ-deficient mice than in WT mice and because of strong virus-specific $CD8^+$ T cell expansion and effector functions, FcεRIγ-deficient mice cleared virus more efficiently culminating in less weight loss and liver damage.

Notably, the cell surface phenotype of robust antiviral $CD8^+$ T cells in in *Fcer1g*$^{-/-}$ mice was found to be similar to that seen during acute infection (Wherry *et al.*, 2007). This finding suggests that FcεRIγ results in $CD8^+$ T cell exhaustion, a hallmark of the chronic virus infection. Thus, FcεRIγ deficiency confers an acute signature mimicry for the LCMV chronically infected mice. A recent study showed that PD-L1 expressing type 1 innate lymphoid cells (ILC1s; authors named this cell type as liver resident NK cells) inhibit T cell functions during LCMV infection in liver immune synapse (Zhou *et al.*, 2019). Nevertheless,

the impact of FcεRIγ in $CD8^+$ T cell exhaustion requires further studies. Even after 55 days of LCMV chronic infection, we could see sustained potent $CD8^+$T cells response in *Fcer1g*$^{-/-}$ mice indicating the durable effect of FcεRIγ on regulating $CD8^+$T cells.

NKp46 directly recognizes the hemagglutinin (HA) proteins of influenza viruses (Mandelboim *et al.*, 2001) and of other viruses such as poxviruses and the Newcastle disease virus (Jarahian *et al.*, 2009). Recently, some non-viral ligands have been elucidated, such as complement factor P and surface protein on healthy pancreatic β cells (Narni-Mancinelli *et al.*, 2017). Moreover, tumor ligands for NCR1 were identified in two metastasis models, the B16F10.9 melanoma (B16) and the Lewis lung carcinoma (D122) (Glasner *et al.*, 2012). Here we found that WT P14 cells express NCR1 ligand which make the virus-specific $CD8^+$ T cells as a main target for NK cell mediated killing via NCR1. However, NCR1 ligand is not affected by FcεRIγ, which rule out the hypersensitivity of $CD8^+$ T cells to NK cell mediated killing in WT mice. *In vivo* killer assays, based on transfer of monoclonal virus-specific $CD8^+$ T cells into recipients challenged with LCMV, revealed that activated LCMV-specific $CD8^+$ T cells were eradicated directly in NCR1- intrinsic FcεRIγ dependent manner.

5.4 FcεRIγ–mediated NK cells role on virus control

The powerful $CD8^+$ T cell response in absence of FcεRIγ resulted in superior viral clearance as mirrored by lower viral titers during chronic infection settings and less immunopathology as noticed by sustained weight and controlled liver enzymes in FcεRIγ deficient mice, suggesting that the faster clearance of virus from antigen-specific $CD8^+$ T cells alleviates the immunopathology in *Fcer1g*$^{-/-}$ mice. Histologic investigation of liver sections retrieved from *Fcer1g*$^{-/-}$ model revealed that the virus is resolved due to robust $CD8^+$ T cells while maintained in WT settings. This could be explained by the fact that, the virus-infected hepatocytes are prone to potent LCMV specific $CD8^+$ T cells in *Fcer1g*$^{-/-}$ mice resulting in virus eradication; whereas, the virus persistence in the WT mice was due to less efficient antiviral $CD8^+$ T cells, this phenomena goes in line with the notion that claims; the viral control and liver damage might be occurred independently in the settings of persistent Hepatitis B virus infection (Maini *et al.*, 2000). More specifically, there is a consensus that, hepatic damage is immune mediated, since the LCMV is somehow hepatotropic, it has been assumed that the recognition of LCMV-infected hepatocytes by LCMV-specific $CD8^+$ T

cells is causing liver damage. Nevertheless, our results show that in the presence of effective LCMV-specific $CD8^+$ T cells response, the inhibition of virus dissemination can be independent of liver damage. When antiviral T cells is unable to control virus replication, it may contribute to liver pathology not only directly but also by causing recruitment of dense infiltrate of nonvirus-specific T cells (Abrignani, 1997). Unlike our findings, Matloubian *et al.,* found that the perforin-deficient $CD8^+$ T cell were enhanced but were unable to clear LCMV infection but were capable of causing immune-mediated damage (Matloubian *et al.*, 1999). In addition, it is plausible that, LCMV can be eradicated from hepatocytes in cytokine-dependent, noncytopathic bystander manner (Guidotti *et al.,* 1999). Since T cells temper cytokine storms by suppressing the innate response (Kim *et al.*, 2007), suboptimal or abortive T cells responses in the WT mice may result in unleashed innate immune responses, causing prolonged production of pro-inflammatory mediators and subsequent liver pathology (Channappanavar *et al.*, 2016).

LCMV was cleared from the circulation and from most of the tested compartments in *Fcer1g*$^{-/-}$ mice within 12 days with a limited existence in kidney due to tubular epithelium anatomical microstructure of the kidney that need IgG to clear the virus rather than $CD8^+$ T cells, and this IgG deposits could be impaired in FcεRIγ-deficient mice. Even for the fraction of IgG fractions that get access into the tubules of that kidney, the pH buffering system could influence the efficiency of IgG. Further, anatomical structure of the kidney might keep the virus "captive and inaccessible" into virus specific- IgG response (Recher *et al.*, 2007).

5.5 *In vivo* depletion studies of NK cells

Previous studies in LCMV settings showed that absence of NK cells was accompanied with the eradication of $CD4^+$ T cells, and subsequently affected $CD8^+$ T cell responses (Waggoner *et al.*, 2011). Further studies showed that NK cell depletion directly influenced $CD8^+$ T cells during LCMV infection (Soderquest *et al.,* 2011). Ablation of NK cells in WT mice led to powerful virus-specific $CD8^+$ T cell response and viral elimination that mimic the T cell immunity and viral control in isotype-treated FcεRIγ-deficient mice. Interestingly, NK cell–depleted *Fcer1g*$^{-/-}$ mice exhibited a negligible to nuanced reduction in $CD8^+$ T cell response compared to isotype-treated *Fcer1g*$^{-/-}$ mice. This could be explained by; there could be a minor role of NK cell- independent FcεRIγ in the early expansion of T cells, and thus the T

cells and/or DCs intrinsic-FcεRIγ might contribute to T cell expansion. Thus, depletion of NK cells in *Fcer1g*$^{-/-}$ animals may overestimate the immune cells, other than NK cells, that have a complex interplay with cytotoxic T cells during chronic LCMV infection in FcεRIγ dependent manner; however, specific studies are necessary for exploring other effects of FcεRIγ on APCs during viral infection.

In conclusion, our observations that NK cells negatively regulate virus-specific CD8^{+} T cell responses via FcεRIγ in a direct manner may not only account for virus infections, but might be elaborated to allo-reactivity or cancer settings or patients with T cell deficiencies, such as those occurring after hematopoietic stem cell transplantation. Deactivation of FcεRIγ (or one of its upstream or downstream signalling molecules) on NK cells might therefore be a suitable avenue to reinvigorate T cell responses in chronic viral infections and cancer. Further, the finding that FcεRIγ plays a role in dampening T cell response *in vivo* has an implication toward developing strategies for adoptive T cell therapy in the treatment of chronic infections and malignancies.

6 SUMMARY

Chronic viral infection is a health condition that afflicts a huge sector of the global population. An effective $CD8^+$ T cell regulation is critical to eradicate the viral infection. Furthermore, NK cells are regarded as innate sentinels and widely defined to regulate the antiviral $CD8^+$ T cells negatively. IFN-I signalling in $CD8^+$ T cells is one of the regulating mediators that renders $CD8^+$ T cells protective against NK cell–mediated killing, however, factors modulating the regulatory functions of NK cells are mainly unknown. Albeit, lymphocytic choriomeningitis virus (LCMV) is NK cells-resistant, it is a prototypical model to investigate the NK-$CD8^+$ T cells crosstalk and it is well-studied model for acute and chronic infections. Herein, we exploited mice that are devoid Fc receptor common gamma chain (FcRγ or FcεRIγ) (*Fcer1g*$^{-/-}$ mice) to address the role of NK cell-intrinsic Fc receptor on shaping the T cell responses in the chronic LCMV settings. We report here that, FcεRIγ deficiency led to potent $CD8^+$ T cell response and efficient control of LCMV, despite the unaffected NK cells quality in FcεRIγ –deficient animals. In addition, we noticed that FcεRIγ is highly expressed intracellularly by NK cells. More specifically, we found that, FcεRIγ-deficient NK cells are not expressing NCR1/NKp46, a unique activating receptor expressed by both resting and activated NK cells. Intriguingly, FcεRIγ was found to stabilize the NCR1 expression via preventing its proteasomal degradation. With the aid of monoclonal LCMV-specific $CD8^+$ T cells transfer and NK cell depletion experiments, we highlight the direct role of NCR1-intrinsic FcεRIγ in eliminating the LCMV-specific $CD8^+$ T cells response.

In summary, our study unravels that lack of FcεRIγ abrogates NKp46 expression on NK cells, and hence compromising their activity on target cells. Thus, NK cell-intrinsic FcεRIγ curtails the $CD8^+$ T cells response in the course of viral infection, converting the acute signature of the disease, whereby the robust $CD8^+$ T cells response and efficient viral control, into a chronic one where the T cells exhaustion, immunopathology and virus persistence.

7 ZUSAMMENFASSUNG

Ein großer Anteil der Weltbevölkerung ist von einer chronischen viralen Infektion betroffen. Eine effektive Regulation der $CD8^+$ T-Zellen ist essenziell für die Bekämpfung einer viralen Infektion. Außerdem gelten natürliche Killerzellen (NK-Zellen) als Wächter des angeborenen Immunsystems, welche die antiviralen T-Zellen negativ regulieren. Der Typ I Interferon (IFN-I)-Signalweg der $CD8^+$-T-Zellen ist einer der regulierenden Mediatoren, welche die $CD8^+$T-Zell Antwort vor dem durch NK-Zellen vermittelten Abtöten schützen. Regulierende Faktoren der natürlichen Killerzellen sind jedoch weitgehend unbekannt. Das Lymphozytäre-Choriomeningitis-Virus (LCMV) ist resistent gegen NK-Zellen und stellt ein typisches Modell zur Untersuchung der Interaktion zwischen den NK-Zellen und den $CD8^+$-T-Zellen dar. In dieser Studie haben wir mit Hilfe eines Mausmodells (*Fcer1g*$^{-/-}$ Mäuse), welchem der Fc-Rezeptor der gemeinsamen Gamma-Kette (FcRγ oder FcεRIγ) fehlt, den Effekt des intrinsischen Fc-Rezeptors der natürlichen Killerzellen auf die Antwort der T-Zellen im Rahmen einer chronischen LCMV-Infektion erforscht. Wir können berichten, dass das Fehlen von FcεRIγ trotz der unveränderten Qualität der natürlichen Killerzellen in den FcεRIγ– Mäusen zu einer starken Immunantwort der $CD8^+$-T-Zellen und einer kontrollierten LCMV-Infektion geführt hat. Außerdem konnten wir bei den NK-Zellen eine hohe intrazelluläre Expression von FcεRIγ feststellen. Darüber hinaus haben wir herausgefunden, dass das Rezeptorprotein NCR1/NKp46, das einen einzigartigen aktivierenden NK-Zell-Rezeptor darstellt und bei ruhenden und aktivierten NK-Zellen exprimiert wird, bei FcεRIγ-defizienten NK-Zellen nicht exprimiert wird. Interessanterweise führte FcεRIγ durch die Hemmung der proteasomalen Degradation von NCR1 zur Stabilisierung der NCR1-Expression. Durch die Übertragung von monoklonalen LCMV-spezifischen $CD8^+$-T-Zellen und die Depletion der NK-Zellen konnten wir die wichtige Rolle des NCR1-intrinsischen FcεRIγ bei der Eliminierung der Immunantwort der LCMV-spezifischen CD8+T-Zellen zeigen. Aufgrund unserer Ergebnisse lässt sich zusammenfassend sagen, dass der Mangel an FcεRIγ zu einem Defekt in der NKp46-Expression in den NK-Zellen führt und somit deren Aktivität beeinträchtigt. Die Immunantwort der $CD8^+$-T-Zellen wird im Verlauf einer LCMV-Infektion durch die Expression des intrinsischen FcεRIγ der NK-Zellen vermindert, wodurch die akute Infektion in eine chronische Infektion mit Erschöpfung der T Zell Antwort und viraler Persistenz umgewandelt wird.

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9 APPENDIX

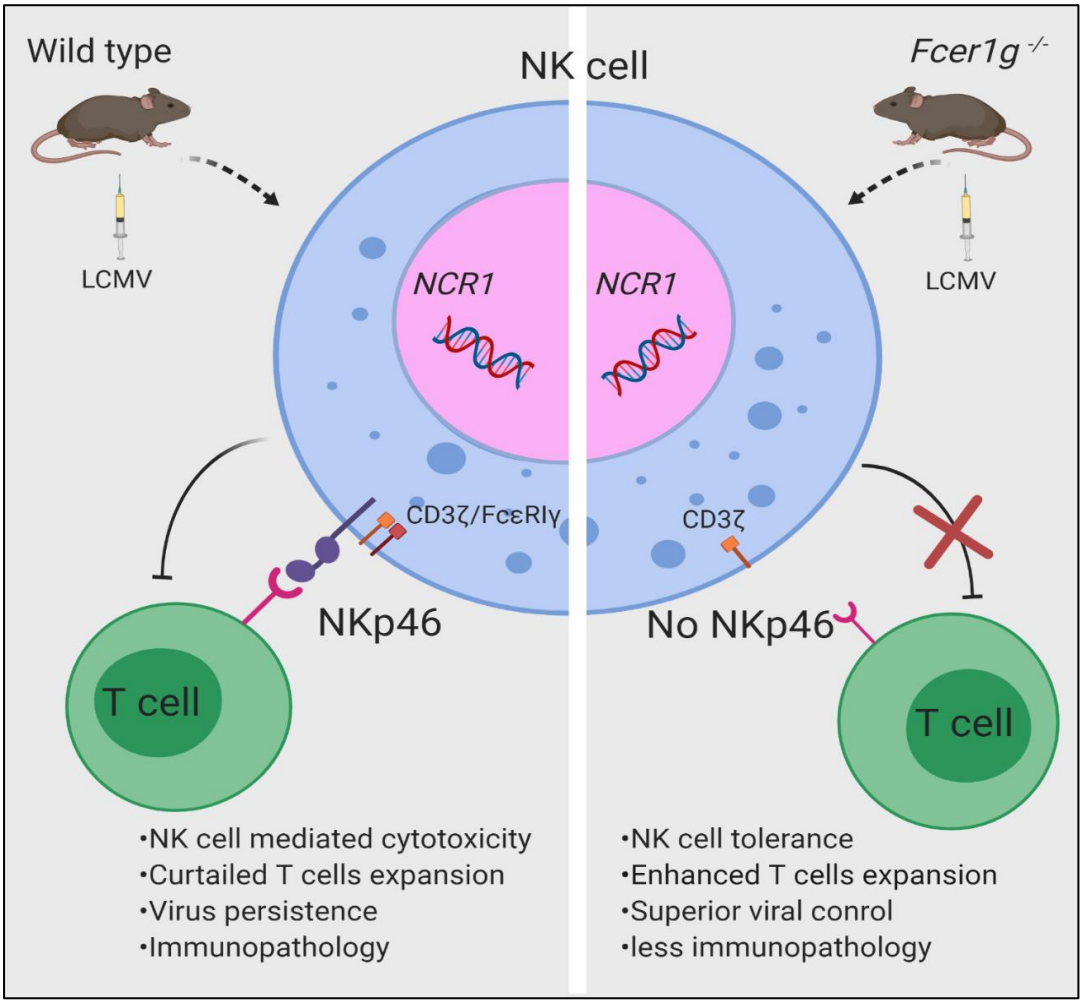
9.1 Abbreviations

7AAD	7-Aminoactinomycin D
Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
ALT	Alanine aminotransferase
APC	Allophycocyanin
APC	Antigen presenting cells
AST	Aspartate transaminase
CD	Cluster of differentiation
CD3ζ	CD3 zeta
CFSE	Carboxyfluorescein succinimidyl ester
CFU	Colony-forming units
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunesorbent assay
FACS	Fluorescence-activated cell sorting
FcR	Fc receptors
FcRγ	Fc receptor, gamma subunit
FCS	Fetal calf serum
FcεRIγ	Fc Fragment Of IgE, High Affinity I, Receptor For; Gamma Polypeptide
FITC	Fluorescein-isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GP	Glycoprotein
GzmB	Granzyme B
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus

HLA	Human leukocyte antigen
HSV	Herpes simplex virus
i.p.	Intraperitoneal
i.v.	Intravenous
IFN	Interferon
IFN-I	Type I interferon
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interlukin
IMDM	Iscove's modified dulbecco's medium
LCMV	Lymphocytic choriomeningitis virus
LCMV-Docile	Lymphocytic choriomeningitis virus strain Docile
LCMV-WE	Lymphocytic choriomeningitis virus strain WE
LDH	Lactate dehydrogenase
LPS	Lipo-polysaccharide
MC57	Mouse fibrosarcoma cell line
MCMV	Murine cytomegalovirus
NCR1	Natural cytotoxicity receptor 1
NK	Natural cytotoxicity
NP	Nucleoprotein
p.i.	Post-infection
p.t.	Post-treatment
PBS	Phosphate buffered saline
PCR	PCR polymerase chain reaction
PE	Phycoerythrin
PeCy7	Phycoerythrin-cyanin-7
PerCP	Peridin-chlorophyll
PFU	Plaque-forming units
PKC θ	Protein kinase C-theta
RNA	Ribonucleicacid
RPM	Rotations per minute
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction

TCR	T-cell receptor
TLR	Toll-like receptor
TRAIL	TNF-related apoptosis-inducing ligand
VSV	Vesicular stomatitis virus
WT	Wild type

9.2 Graphical abstract



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9.3 List of figures

Introduction

Results

9.4 Supplementary table

Antibody	Conjugate	Clone	Source
7-AAD viability staining solution	-------	--------	Biolegend
AffiniPure goat anti-rabbit IgG (H+L)	FITC	LG.7F9	Jackson ImmunoResearch
Annexin V	PE	---------	Biolegend
Anti-CD3ζ antibody	FITC	H146-968	Abcam
Anti-FcεRI antibody, γ subunit	FITC	polyclonal	Merck
Anti-human/mouse granzyme B antibody	Pacific Blue	GB11	Biolegend
CD107a (LAMP-1)	FITC	1D4B	Biolegend
CD253 (TRAIL)	PE/Cy7	N2B2	Biolegend
CD314 (NKG2D)	FITC	C7	Biolegend
CD335 (NKp46)	PE/Cy7	29A1.4	eBioscience
CD335 (NKp46)	PE	29A1.4	Biolegend
Ly-6A/E (Sca-1)	APC/Cy7	D7	Biolegend
Anti-mouse NK1.1	APC	PK136	Biolegend
Anti-mouse NK1.1	APC	PK136	Biolegend
Anti-PKCθ antibody	-----	2F1	Abcam
B220	APC	RA3-6B2	eBioscience
CD115	PE	AFS98	eBioscience
CD11b	Q-dot	M1/70	BD bioscience
CD11c	PE/Cy7	N418	eBioscience
CD11c	Brilliant violet 510	N418	Biolegend
CD11c	Q-dot	N418	ebioscience
CD19	Q-dot	1D3	eBioscience
CD27	FITC	LG.3A10	Biolegend
CD27	APC-eFluor 780	CXCR3-173	eBioscience
CD279 (PD-1)	FITC	RMP1-30	eBioscience
CD4	APC	GK1.5	eBioscience
CD40	FITC	HM40-3	Biolegend
CD45.1	PE	A20	ebioscience
CD45.2	V500	104	BD bioscience
CD69	PE	H1.2F3	eBioscience
CD86	PE/Cy7	GL1	BD
CD8a	PE/Cy7	52-6.7	invivogen
CD90.1 (Thy-1.1)	APC	Polyclonal	eBioscience
F4/80	APC	BM8	eBioscience
Armenian hamster IgG isotype	FITC	HTK888	Biolegend
IFN-γ monoclonal antibody	PE	XMG1.2	eBioscience
KLRG1 monoclonal antibody	PE-eFluor 610	2F1	eBioscience
Ly-49H monoclonal antibody	FITC	3D10	eBioscience
Ly6C	Pacific blue	HK1.4	eBioscience
Ly6G	PerCP/Cy5.5	RB6-8C5	invivogen

MHC II	PE	M5/114.15.2	eBioscience
MHC II	APC	M5/114.15.2	invitrogen
NCR1	Alexa Fluor 700	29A 1.4	R&D Systems
Perforin monoclonal antibody	PE	EBioOMAK-D	eBioscience
Rat IgG2a κ isotype control	PE	eBR2a	eBioscience
Rat IgG2a, κ isotype ctrl antibody	PE/Cy7	RTK2758	Biolegend
TCR V alpha 2	PE	B20.1	Thermofisher
TCR-β monoclonal antibody	PE	H57-597	eBioscience
TNF-α monoclonal antibody	APC	MP6-XT22	eBioscience

Abbreviations: 7-AAD, 7-Aminoactinomycin D; APC, antigen-presenting cell; FITC, fluorescein isothiocyanate; PerCP, Peridinin-chlorophyll proteins ; IgG, immunoglobulin G; PE, phycoerythrin; TCR, T-cell receptor; TRAIL, TNF-related apoptosis-inducing ligand; PKC, protein kinase C theta; PE-Cy7: Phycoerythrin-Cyanine7

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11 CURRICULUM VITAE

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