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Using Mice with Conditional Deletion of the IFN γ Receptor to Identify the Cellular
Targets of IFN γ during Development of Anti-*Listeria* Immune Responses.

By

Sang Hun Lee

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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ABSTRACT OF THE DISSERTATION

Using Mice with Conditional Deletion of the IFN γ Receptor to Identify the Cellular Targets of IFN γ during Development of Anti-*Listeria* Immune Responses.

By

Sang Hun Lee

Doctor of Philosophy in Biology and Biomedical Sciences (Immunology)

Washington University in St. Louis, 2012

Professor Robert D. Schreiber, Mentor

Host responsiveness to IFN γ is critical for resolution of *Listeria* infection, but the identities and roles of IFN γ responsive cells that initiate this process remain unclear. In this thesis, unique mice displaying conditional loss of the IFN γ receptor (*Ifngr*^{ff} mice) in different tissues have been generated and used to explore the role of this cytokine in initiating the anti-*Listeria* response.

Whereas *Ifngr*^{ff} mice displayed normal cellular levels of IFNGR1 and normal resistance to *Listeria* infection, *Vav-icre*⁺*Ifngr*^{ff} mice lacking IFN γ responsiveness selectively in hematopoietic cells exhibited highly increased susceptibility to *Listeria* infection comparable to that of *Ifngr*^{-/-} mice. In contrast, *Itgax-cre*⁺*Ifngr*^{ff} mice lacking IFN γ responsiveness selectively in dendritic cells expressing CD8 α and/or CD103 displayed increased susceptibility to *Listeria* infection. This phenotype was traced back to

a defect in the capacity of IFN γ unresponsive CD8 α^+ DCs to produce IL-12. The IFN γ required for priming CD8 α^+ DCs for optimal IL-12 production is derived from TNF α -activated NK/NKT cells. These mice survived *Listeria* infection due to a second wave of IL-12 produced by other myeloid cells.

Next, *IfngrI^{ff}* mice were bred with *LysM-cre* mice to obtain myeloid cell specific *IfngrI* deletion. *LysM-cre⁺IfngrI^{ff}* mice lacking IFN γ responsiveness selectively in both peritoneal macrophages and neutrophils succumbed to *Listeria* infection. Surprisingly, adoptively transferred WT neutrophils into *LysM-cre⁺IfngrI^{ff}* mice seemed to completely rescue *LysM-cre⁺IfngrI^{ff}* mice whereas *IfngrI^{-/-}* neutrophils did not. Thus, these results reveal that neutrophils are also one of the important targets of IFN γ for *Listeria* resolution.

This study thus demonstrates an early acting, IFN γ driven cytokine and cellular cascade involving NK/NKT and CD8 α^+ DCs leads to rapid production of IL-12 that ultimately leads to activation of myeloid cells including macrophages and neutrophils in IFN γ -rich cytokine environment.

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LIST OF ABBREVIATIONS

| | |
|--------|-------------------------------------------|
| BM | Bone Marrow |
| CD | Cluster of Differentiation |
| CFU | Colony Forming Unit |
| CXCR3 | Chemokine (CXC motif) Receptor 3 |
| DC | Dendritic Cell |
| DTR | Diphtheria Toxin Receptor |
| EGFP | Enhanced Green Fluorescence Protein |
| EDTA | EthyleneDiamineTetraAcetate |
| ES | Embryonic Stem |
| FACS | Fluorescence Activated Cell Sorting |
| HBSS | Hank's Buffered Salt Solution |
| HKLM | Heat Killed <i>Listeria Monocytogenes</i> |
| IFNAR1 | InterFeroN Alpha/beta Receptor 1 |
| IFNGR1 | InterFeroN Gamma Receptor 1 |
| IL | InterLeukin |
| iNOS | Inducible Nitric Oxide Synthase |
| IP | IntraPeritoneal |
| ITGAX | InTeGrin Alpha X |
| IV | IntraVenous |
| LysM | Lysozyme M |
| mAb | Monoclonal Antibody |

| | |
|-----------|--------------------------------------------------|
| MFI | Mean Fluorescence Intensity |
| MZM | Marginal Zone Macrophage |
| MMM | Metallophilic Marginal zone Macrophage |
| NK | Natural Killer |
| NKT | Natural Killer T |
| NO | Nitric Oxide |
| PALS | PeriArteriolar Lymphoid Sheaths |
| PBL | Peripheral Blood Leukocyte |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| pDC | Plasmacytoid Dendritic Cell |
| PEC | Peritoneal Exudate Cell |
| RAW 264.7 | Mouse Leukemic Monocyte Macrophage Cell Line |
| RBC | Red Blood Cell |
| RT | Room Temperature |
| SCID | Severe Combined ImmunoDeficiency |
| SEM | Standard Error of the Mean |
| STAT | Signal Transducer and Activator of Transcription |
| TipDC | TNF α and iNOS Producing Dendritic Cell |
| TNF | Tumor Necrosis Factor |
| TNFR | Tumor Necrosis Factor Receptor |
| WT | Wild Type |

CHAPTER 1

Introduction

IFN γ is crucial for immunity against intracellular bacterial pathogens such as *Listeria Monocytogenes*. IFN γ coordinates a large array of immunological responses through transcriptional regulation of genes which are relevant to both innate and adaptive immunities. IFN γ mediated cellular responses are integrated with other pathogen associated cytokines, such as TNF α and IL-12. Cellular effects of IFN γ include pathogen recognition, promotion of antigen processing and presentation, regulation of cellular proliferation and apoptosis, induction of microbicidal effector functions, and direction of leukocyte trafficking. However, it remains to be addressed which components of the innate, adaptive or non-hematopoietic compartments require IFN γ sensitivity in order to resolve *Listeria* infection.

Type II interferon (IFN γ)

Interferons (IFNs) were originally described as cytokines capable of protecting host cells from pathogens (Farrar and Schreiber, 1993). The members of IFNs have been divided into two different groups based on their cellular source, genetic structure, and biological activities. Type I IFN is induced primarily by viral infection of cells and has been subdivided into two classes, IFN α and IFN β . In contrast, Type II IFN is induced by various pro-inflammatory stimuli and consists of a single member, IFN γ . IFN γ has no genetic and structural similarity with Type I IFNs; IFN γ is located in different

chromosome and its protein structure is distinct from that of IFN α and IFN β . The crystallography of IFN γ confirmed its dimeric structure which is associated in an antiparallel orientation (Ealick et al., 1991).

IFN γ binds to a distinct cell surface receptor, IFN γ receptor (IFNGR) and primarily signals through the JAK-STAT pathway, a pathway used by various cytokines, growth hormones, and hormones to regulate gene expression (Bach et al., 1997). Functional IFN γ receptor is composed of a ligand binding subunit, IFNGR1, and a signal transducing subunit IFNGR2. IFNGR1 is constitutively expressed on the surface of nearly all types of cells whereas IFNGR2 appears to be expressed at extremely low levels and regulated by external stimuli. We previously demonstrated that the IFN γ unresponsiveness in a subset of T cells (Th1) is due to a lack of cellular expression of IFNGR2 (Bach et al., 1995). Thus, the regulation of IFNGR2 gene becomes a limiting factor for IFN γ responsiveness in certain cells. The human IFNGR1 is encoded by a 30kb gene located on chromosome 6 (Aguet et al., 1988), while the murine homologue is a 22kb gene present on chromosome 10 (Gray et al., 1989; Hemmi et al., 1989). The human IFNGR2 has been localized to chromosome 21 and the murine homologue resides on chromosome 16 (Hemmi et al., 1994; Soh et al., 1994).

Neither IFNGR chains have intrinsic kinase/phosphatase activity. Thus, the intracellular domain of IFNGR1 has a $_{266}\text{LPKS}_{269}$ binding motif for the Janus tyrosine kinase 1 (JAK1), and the IFNGR2 intracellular region contains a binding motif ($_{263}\text{PPSIPLQIEEYL}_{274}$) for JAK2 (Bach et al., 1996; Greenlund et al., 1994). Both IFNGR subunits are constitutively associated with inactive form of JAKs through their

motifs. The binding of IFN γ , a homo-dimeric ligand, to the cells in 2:2 stoichiometry induces assembly of the complete receptor complex containing two IFNGR1 and two IFNGR2. In this complex, JAK2 is auto-phosphorylated/activated and JAK1 is subsequently phosphorylated by activated JAK2. The activated JAK1 phosphorylates a key tyrosine residue (Y440) of ₄₄₀YDKPH₄₄₄ motif within the cytoplasmic domain of each IFNGR1 to form two docking site for the SH2 domains of STAT1 (Farrar et al., 1992; Greenlund et al., 1994). Two STAT1 molecules are then brought into close proximity with receptor-associated activated JAK enzymes. The receptor recruited STAT1 pair is phosphorylated at Y701 probably by JAK2, leading STAT1 dimerization and dissociation from the receptor. The dissociated STAT1 homodimer translocates into the nucleus and binds to transcriptional regulatory elements such as IFN γ activation site (GAS) element to initiate or suppress transcription of IFN γ -regulated genes. The gene transcription induced by IFN γ occurs within 15-30 minutes of IFN γ treatment (Kerr and Stark, 1991). Many genes induced by IFN γ are in fact transcription factors including IRF1 which is able to initiate the second wave of transcription.

IFN γ effects on a wide range of target cells, and its pleiotropic actions have been well studied (Farrar and Schreiber, 1993). These include various host defense mechanisms against infectious agents and tumor development. IFN γ can exert antiproliferative effects on a variety of normal and neoplastic cells. IFN γ is also acknowledged to play an immune-regulatory roles. IFN γ is one of the major cytokines responsible for enhancing antigen processing and presentation in part by up-regulating MHC class I expression and inducing MHC class II on antigen presenting cells. IFN γ has

also been shown to be the major cytokine responsible for activating mononuclear phagocytes to clear pathogens. In addition, IFN γ regulates antibody responses by modulating Ig class switching recombination. Finally, IFN γ regulates the production of a variety of other immune-regulatory or pro-inflammatory cytokines such as IL-12 and TNF α .

Listeria Monocytogenes

Listeria monocytogenes is a gram-positive intracellular bacterium that can cause significant disease in neonates, the elderly, and immunocompromised individuals (Pamer, 2004). As a food-borne pathogen, the natural route of *Listeria* infection is through the gastrointestinal tract. *Listeria* is capable of invading intestinal epithelial cells by inducing cytoskeletal changes and membrane extensions (Cossart and Lecuit, 1998). This ‘engulfing’ requires the interaction between internalin A on the surface of *Listeria* and E-cadherin on the surface of epithelial cells. Mice are relatively more resistant to intestinal *Listeria* infection than humans because of a particular amino acid difference between human (Pro₁₆) and mouse E-cadherin (Glu₁₆) renders the murine form of the protein insensitive to internalin A-mediated invasion (Lecuit et al., 2001). Once *Listeria* invades the epithelial layer, they are rapidly disseminated into spleen and liver through the blood stream. In the liver, *Listeria* infects hepatocytes through the interaction of internalin B and the Met receptor tyrosine kinase that is expressed at the surface of hepatocytes (Shen et al., 2000). In spleen and liver, *Listeria* is rapidly internalized by splenic and hepatic phagocytes. After entering the phagosomes, *Listeria* penetrates the vacuolar membrane

and enters the cytoplasm through secreting listeriolysin O (LLO), the pore forming toxin (Bielecki et al., 1990). LLO-mediated invasion of the cytosol is important to induce protective immunity because LLO deficient strain of *Listeria* fails to induce a protective T cell mediated response (Berche et al., 1987). In the cytoplasm, *Listeria* proliferates and induces actin polymerization by expressing actin assembly inducing protein (ActA) in a polarized manner, which generates bacterial movement and finally causes cell-to-cell infection spreading (Tilney and Portnoy, 1989).

Immunity to *Listeria* infection

The mouse model of *Listeria* infection has been extensively studied for understanding the mechanisms of host resistance to an intracellular pathogen. Since George Mackaness first developed the systemic murine infection model for *Listeria* (Mackaness, 1962), most studies of *Listeria* infection have used either intraperitoneal or intravenous inoculation of bacteria. Thus, our current knowledge of immune responses against *Listeria* infection is mostly based on systemic infection although the natural route of *Listeria* infection is ingestion of bacteria and subsequent uptake of them by intestinal epithelial cells.

The innate immune response to *Listeria* involves a complex interaction of various cytokines and innate immune cells such as macrophages. By using a neutralizing mAb for IFN γ , the activation of macrophages by IFN γ was shown to be critical for *in vivo* clearance of bacteria from the infected animal (Buchmeier and Schreiber, 1985). The importance and effectiveness of innate immunity to *Listeria* infection was further proven

using SCID (Severe combined immunodeficiency) mice (Bancroft et al., 1989; Edelson and Unanue, 2000). Mice which are devoid of T and B cells were shown to be surprisingly effective in controlling initial *Listeria* infection although the complete sterilization of bacterial infection requires adaptive immunity. Subsequent studies using SCID mice revealed that NK cells were responsible for early production of IFN γ and that the IFN γ produced by NK cells activated microbicidal activity in macrophages thus providing the host with a rapid ability to control the initial stages of *Listeria* infection (Bancroft et al., 1989; Tripp et al., 1993; Wherry et al., 1991). This observation stimulated mechanistic studies that employed neutralizing/depleting antibodies and gene-targeted mice to define many of the components of innate immunity that participates in the early host response to *Listeria* (Bancroft et al., 1989; Brombacher et al., 1999; Dunn and North, 1991; Tripp et al., 1994). For example, activation of macrophages is clearly critical for immunity to *Listeria* because antibody-mediated neutralization/genetic ablation of IFN γ /IFN γ R or pharmacological inhibition/genetic ablation of iNOS (inducible nitric oxide synthase) and NADPH oxidase results in markedly increased susceptibility to *Listeria* infection (Buchmeier and Schreiber, 1985; Dalton et al., 1993; Huang et al., 1993; Shiloh et al., 1999).

Still other studies revealed that IFN γ is produced throughout the course of infection and plays a key role in driving the development and effector functions of the Type I T cell response (Th1 CD4⁺ T cells and CD8⁺ T cells) responsible for development of the sterilizing immunity (Macatonia et al., 1993; Szabo et al., 1997). Together this work resulted in a detailed understanding of the molecular and cellular events that occur

during host responses to *Listeria* infection (Unanue, 1997). Early in infection, low levels of TNF α and IL-12 are thought to stimulate production of low levels of IFN γ by NK cells. This early-appearing IFN γ then activates microbicidal activity in macrophages thereby providing the host with a mechanism to control infection until sterilizing, adaptive immunity can develop. Subsequently, the IFN γ - and IL-12-rich cytokine environment promotes induction of a Type I T cell response that produces even more IFN γ and induces the cellular effector functions needed to produce sterilizing immunity to the bacteria.

Although early resistance to *Listeria* infection is largely attributed to the production of TNF α and IL-12 by macrophages resulting in initial production of IFN γ , it should be noted that the previous studies were largely based on macrophages which were obtained from a lavage of SCID mouse peritoneal cavities. It is now clear that within a few minutes of infection, *Listeria* is taken up by various phagocytic cells in spleen and other organs (Waite et al., 2011). In the spleen, blood-borne pathogens are filtered out of blood stream by resident cells in the marginal zone and red pulp (Aoshi et al., 2009). Splenic resident phagocytes include marginal zone macrophages, metallophilic marginal zone macrophages, neutrophils, F4/80⁺ red pulp macrophages, CD8 α ⁺ dendritic cells, and CD4⁺ dendritic cells. Therefore, with regards to the production of TNF α and IL-12 *in vivo*, it remains incompletely understood which one(s) among various resident phagocytes is the major producer of these cytokines.

IFN γ for *Listeria* resolution

Early production of IFN γ in the host is crucial for the timely resolution of *Listeria* infection, as demonstrated by studies using a neutralizing antibody to IFN γ (Buchmeier and Schreiber, 1985) and subsequently using mice lacking either IFN (Dalton et al., 1993), IFNGR1 - the major ligand binding chain of the IFN γ receptor (Huang et al., 1993), or STAT1, the major transcription factor that mediates IFN γ receptor signaling (Goldszmid et al., 2012; Meraz et al., 1996). However, both the *in vivo* cellular sources of IFN γ and in particular the IFN γ -responsive cells that initiate and regulate protective responses have been difficult to identify.

NK cells have been thought to be the major producer of IFN γ after *Listeria* infection (Unanue, 1997). IFN γ production by NK cells can be stimulated by several cytokines including TNF α from macrophages in combination with IL-12 (Tripp et al., 1993), trans-presented IL-15 by dendritic cells (Lucas et al., 2007), and Type I IFN (Biron et al., 1999). Humann and Lenz demonstrated that the activation of NK cells in *Listeria*-infected mice depends on IL-18 as well as IL-12 (Humann and Lenz, 2010). Direct contact between DC and NK cells is also required for priming NK cells, resulting in enhanced cytotoxicity and IFN γ production (Andrews et al., 2003; Gerosa et al., 2002). The spatiotemporal analysis of potential NK and DC interactions in *Listeria*-infected spleens showed that CD11c⁺ DCs orchestrate the clustering of CD11b⁺ myeloid cells into periarteriolar lymphoid sheaths (PALS) and the recruitment of NK cells to the outer edges of these clusters where the production of IL-12 and IL-18 was detected (Kang et al.,

2008). Hence, it seems probable that contact with *Listeria*-infected DCs directly activates NK cells to produce IFN γ in PALS.

Memory CD8⁺ T cells are another viable candidate as an important source of IFN γ . Berg et al. first demonstrated that memory CD8⁺ T cells have the potential to secrete IFN γ after *Listeria* infection without TCR engagement in a few days of infection (Berg et al., 2003, 2005). One study which used a *Salmonella Typhimurium* infection model demonstrated that activation of inflammasomes leads to the release of IL-18 by CD8 α ⁺ DCs, which directly induces memory CD8⁺ T cells to produce IFN γ (Kupz et al., 2012). Finally, splenic CD8 α ⁺ DCs themselves were also reported as a potential source of IFN γ in response to *Listeria* infection in the absence of NK cells (Ohteki et al., 1999).

In contrast to defining the sources of IFN γ , it has been particularly difficult to identify the IFN γ responsive cells that are responsible for initiating the anti-*Listeria* response since nearly every cell in the host expresses an IFN γ receptor (Bach et al., 1997). It is likely that there are many direct cellular targets of IFN γ that participate in the anti-*Listeria* response (Schroder et al., 2004). An early study used radiation bone marrow (BM) chimera approaches to demonstrate that IFN γ receptor (IFN γ R) expression in the hematopoietic compartment was required for resistance to *Listeria* infection (Yap and Sher, 1999). This initial demonstration prompted attempts to identify the *in vivo* targets of the antibacterial actions of IFN γ . Because macrophages are known critical targets of IFN γ , the Schreiber lab generated transgenic mice in which a dominant negative IFN γ receptor mutant (IFNGR1. Δ IC) was targeted to resident peritoneal macrophages using the human lysozyme promoter and identified a subset of macrophages that were key

mediators of the IFN γ -dependent anti-*Listeria* response (Dighe et al., 1995). Recently, a study using transgenic mice that express IFNGR1. Δ IC in CD68⁺ cells showed the additional importance of IFN γ -mediated macrophage activation for control of *Toxoplasma gondii* infection (Lykens et al., 2010). These studies validate the use of transgenic mice as a means of selectively eliminating IFN γ responsiveness in macrophages. Additional studies in which specific tissues are rendered IFN γ -unresponsive could provide valuable insight into the mechanisms underlying control of intracellular pathogens. However, the use of tissue targeted overexpression of dominant negative IFNGR1 mutants is not an ideal approach since extremely high expression of the mutant receptor is required in order to suppress normal signaling by the wild type IFN γ R (Dighe et al., 1995).

Subsets of murine dendritic cells

Since Ralph Steinman first identified dendritic cells as potent immune cells which are involved in the induction of immune responses (Steinman and Cohn, 1973), numerous studies support the idea that dendritic cells are extremely efficient antigen presenting cells. For example, after CD11c (*itgax*; integrin alpha X) was identified as DC-selective marker (Metlay et al., 1990), the generation of CD11c-DTR mice which depletes DCs relatively selectively by single injection of diphtheria toxin was used to first demonstrate that the priming of anti-*Listeria* immunity depends on CD11c⁺ dendritic cells (Kang et al., 2008).

DCs in mouse secondary lymphoid organs can be subdivided into two groups, conventional DCs (cDC) and plasmacytoid DCs (pDCs). One of the key roles of pDCs is the production of Type I IFN. It remains unclear whether pDCs perform an antigen presentation function (Villadangos and Young, 2008). Conventional DCs can be subdivided into resident DCs and migratory DCs. Resident DCs can be further categorized into three subsets on the basis of their expression of CD4 and CD8 α , namely CD4⁺ DCs, CD8 α ⁺ DC, and double negative DCs (Vremec et al., 2000). Many studies have led to the concept that lymphoid organ resident CD8 α ⁺ DCs are specialized for cross-presenting antigen (Heath and Carbone, 2009). When purified DC subsets from *Listeria*-infected mice were incubated with antigen-specific CD8⁺ T cells, CD8 α ⁺ DC were the only cells that cross-presented antigens to prime naïve CD8⁺ T cells (Belz et al., 2005). Recently, Hildner *et al.* generated *Batf3*-deficient mice that lacked lymphoid-resident CD8 α ⁺ DCs (and also CD103⁺ DCs) without other abnormalities (Hildner et al., 2008). As expected, *Batf3*-deficient mice were not capable of cross-presenting antigens and priming CD8⁺ T cell responses to tumor antigens and intracellular pathogens such as West Nile and Sendai viruses *in vivo*.

Migratory DCs in dermis are currently divided into at least three distinct subsets, Langerhans cells, CD103⁺CD11b⁻Langerin⁺ DCs, and CD103⁻CD11b⁺ Langerin⁻ DCs. Recent data described the existence of CD103⁺CD11b⁻ DCs and CD103⁻CD11b⁺ DCs in most peripheral organs including lung, liver, and kidney (Ginhoux et al., 2009). Since Langerhans cells were the first DCs to be observed in skin sections by Paul Langerhans (Romani et al., 2003), they were thought to have the classical phenotype of DCs.

However, after careful analysis of the function and location of langerin-expressing DCs, three independent groups identified a novel subset of DCs that is langerin positive and distinct from Langerhans cells and CD11b⁺ dermal DCs (Bursch et al., 2007; Ginhoux et al., 2007; Poulin et al., 2007). Extensive characterization of this novel dermal DC population showed that they were bone marrow derived, radio-sensitive and expressed the CD103 integrin and were therefore distinct from classical radio-resistant Langerhans cells. Most importantly, CD103⁺ DCs are capable of migrating from peripheral tissues such as skin to secondary lymphoid tissues and cross-presenting antigen (del Rio et al., 2007; GeurtsvanKessel et al., 2008). Thus, the migratory CD103⁺ DCs and the lymphoid tissue-resident CD8 α ⁺ DCs have phenotypic and functional similarities including expression of langerin, absence of CD11b expression, and the capacity to cross-present antigens to prime CD8⁺ T cell responses.

Importance of dendritic cells during *Listeria* infection

Recently, much attention has focused on the role of dendritic cells (DCs) in the development of *Listeria* infection. DCs are the primary cell type that senses, ingests, and presents exogenous antigens from pathogens to initiate the adaptive immune response. CD8 α ⁺ DCs appear more potent than other DC subsets in processing and cross-presenting exogenous antigens on surface MHC class I (Belz et al., 2005; Dudziak et al., 2007; Jung et al., 2002). Their efficiency in cross-presentation is partly due to the delayed phagosomal acidification relative to other phagocytes such as neutrophils, macrophages, and other DC subsets (Savina et al., 2009). Conceivably, this delay helps CD8 α ⁺ DCs

maintain higher antigen levels for cross-presentation, resulting in increased susceptibility of CD8 α^+ DCs to pathogens. Actually, *Listeria* was shown to exploit this intrinsic susceptibility of CD8 α^+ DCs as part of bacterial propagation in spleen. The studies using CD11c-DTR mice revealed that mice depleted of CD11c $^+$ cells lack productive *Listeria* infection in the spleen (Kang et al., 2008; Neuenhahn et al., 2006). Furthermore, using *Batf3* $^{-/-}$ mice, that selectively lack CD8 α^+ DCs, it was possible to demonstrate this subset of DCs was required for establishing *Listeria* infection in the spleen (Edelson et al., 2011; Hildner et al., 2008). These findings support a scenario in which migratory CD8 α^+ DCs carry *Listeria* from their entry point in the splenic marginal zone to the T cell-rich PALS, where *Listeria* then multiply in the ensuing 12-24 hours (Aoshi et al., 2008; Muraille et al., 2005). In addition, Edelson *et al.* demonstrated that hepatic resistance to *Listeria* infection is due to the absence of hepatic CD103 $^+$ DCs in *Batf3* $^{-/-}$ mice. Perhaps CD103 $^+$ DCs in hepatic sinusoids efficiently capture *Listeria* and transport them into the hepatic parenchyma for productive liver infection.

Another important role of CD8 α^+ DCs has been reported to be regulating hierarchical clustering/activation of innate cells such as NK cells, neutrophils, and monocytes (Kang et al., 2008). By challenging mice having a CD11c-cre transgene and a diphtheria toxin A gene downstream of a flox-stop-flox into ROSA26 locus with *Listeria*, Kang et al. showed *in vivo* depletion of DCs completely abrogated innate cell clustering and subsequent cytokine production such as IL-12 and IFN γ around infection foci in the white pulps of spleen. Therefore, CD8 α^+ DCs facilitate the entry and expansion of *Listeria* within clusters of innate cells in PALS. The precise mechanism by which CD8 α^+

DCs coordinate innate cell clustering at early times after infection remains to be defined. It is not clear whether the complete abrogation of innate cell clustering in CD11c-DTR mice is due to the absence of CD8 α ⁺ DCs or merely the absence of *Listeria* infection caused by depletion of essential bacterial carriers, CD8 α ⁺ DCs.

Interleukin-12 for *Listeria* resolution

IL-12 is a heterodimer formed by a 35-kDa light chain (p35/IL-12 α) and a 40-kDa heavy chain (p40/IL-12 β). Recently, it was found that p40 associates not only with p35 to form IL-12, but also with p19 to form a new heterodimeric cytokine known as IL-23 (Oppmann et al., 2000). The IL-12 receptor is composed of two polypeptides, IL-12R β 1 and IL-12R β 2. The IL-12R β 2 subunit functions as a signal-transducing component, which induces the phosphorylation and activation of Janus family kinase JAK2 and TYK2. Activated JAK2 and TYK2 were shown to phosphorylate STAT1, 3, 4, and 5 (Presky et al., 1996). However, the specific cellular effects of IL-12 are due mainly to its activation of STAT4 because STAT4 deficient mice showed the identical phenotype to mice which lack IL-12p40 (Thierfelder et al., 1996).

Interleukin-12 (IL-12) was first described independently as a potent NK activating factor and cytotoxic lymphocyte maturation factor (Kobayashi et al., 1989; Stern et al., 1990). It has since been extensively studied for its pro-inflammatory ability to stimulate IFN γ production from NK cells and T cells and its immune-regulatory ability to promote the development of the Th1 response (Chan et al., 1991; Hsieh et al., 1993). The importance of IL-12 as an IFN γ inducer has been appreciated not only in its high

efficiency even in low concentration, but also in its synergy with other stimulatory factors such as IL-2, TCR engagement, and TNF α (Chan et al., 1992; Chan et al., 1991). In addition, IL-12 also regulates the contraction phase of CD8⁺ T cell response and the generation of memory response (Pearce and Shen, 2007; Takemoto et al., 2006).

IL-12 is essential for resistance to sublethal doses of *Listeria* by inducing optimal IFN γ production in NK cells (Brombacher et al., 1999; Tripp et al., 1994). IL-12p35^{-/-} mice showed increases in the number and size of granulomatous lesions even at low doses of *Listeria* (≤ 1000 organisms). DCs and phagocytes including monocytes, macrophages, and neutrophils are the main producers of IL-12 in response to microbial stimulation (Trinchieri, 2003). However, *in vivo* depletion of resident cDC using CD11c-DTR prior to *Toxoplasma gondii* infection has been shown to significantly reduce systemic IL-12 production (Liu et al., 2006). A subsequent study using *Batf3*^{-/-} mice which genetically lack CD8 α ⁺ DCs demonstrated that resident CD8 α ⁺ DCs are the main producers of IL-12 at least in *T.gondii* infection (Mashayekhi et al., 2011). CD11c⁺MHCII⁺ DCs derived from Ly6C⁺ inflammatory monocytes were recently also shown to be another source of IL-12 after *T.gondii* infection, and this production of IL-12 depends on IFN γ from NK cells (Goldszmid et al., 2012). In *Listeria* infection, both resident CD8 α ⁺ DCs and monocyte-derived DCs were shown to produce IL-12 (Kapadia et al., 2011; Mitchell et al., 2011; Zhan et al., 2010). Although both resident CD8 α ⁺ DCs and monocyte-derived DCs can produce IL-12 during *Listeria* infection, the temporal relationship of their participation in *in vivo* IL-12 production has, until now, not been studied.

Importance of neutrophils during *Listeria* infection

Neutrophils have long been thought to be an important innate cell for clearance of *Listeria*, appearing at sites of infection within the first 24 hours. An early study used a monoclonal antibody (RB6-8C5) specific for granulocyte receptor 1 (GR-1) to deplete these cells, and demonstrated that mice depleted of neutrophils succumbed to an early, lethal infection with 10-100 fold increased bacterial burdens in the liver (Conlan and North, 1994). It has been suggested that neutrophils directly lyse *Listeria* infected hepatocytes and actually engulf apoptotic hepatocytes (Rogers et al., 1996). Neutrophils were also suggested to represent sources of pro-inflammatory cytokines such as IFN γ and TNF α which are required for the clearance of *Listeria* (Grivennikov et al., 2005; Yin and Ferguson, 2009).

At the time of these conclusions, however, it was not appreciated that RB6-8C5 recognizes both Ly6G and Ly6C and accordingly depletes multiple types of cells (Nagendra and Schlueter, 2004). Ly6G expression is restricted to neutrophils, whereas Ly6C is expressed by neutrophils, inflammatory monocytes, and subsets of CD8⁺ T cells (Fleming et al., 1993). Recent studies demonstrated that selective depletion of neutrophils by the 1A8 anti-Ly6G mAb resulted in 10-1000 fold increased infection in livers and with a higher dose of *Listeria*, increased bacterial burdens in the spleens were also observed (Carr et al., 2011). In contrast, Shi et al. failed to see significant difference in bacterial burdens after 1A8 treatment (Shi et al., 2011). This may be due to the same day treatment of mAb with *Listeria* infection which causes the delayed deletion of neutrophils.

Although neutrophils are one of the first cells to respond to *Listeria* infection, the obsolete concept of the neutrophils as terminally differentiated, short-lived, and not transcriptionally active cells has hampered researches on non-phagocytic roles of neutrophils. Most investigations into the functions of IFN γ in innate immunity have also overlooked the significance of IFN γ on the functions of neutrophils (Ellis and Beaman, 2004). However, *in vitro* treatment of neutrophils from peripheral blood with IFN γ elicits a variety of responses such as oxidative burst, cytokine and chemokine production, antigen presentation, phagocytosis, cytotoxic effects, and chemotaxis. Thus, non-traditional roles of neutrophils which respond to cytokine stimuli such as IFN γ via gene expression and regulation may be critical for *Listeria* resistance.

Summary of current findings

In this study, I hypothesized that IFN γ might act on the CD8 α^+ DC subset to initiate pathways leading to *Listeria* resistance. To investigate this issue, I generated mice with a floxed *Ifngr1* gene (*Ifngr1^{ff}* mice) on a pure C57BL/6 genetic background and then bred them to either *Vav-icre* or *Itgax-cre* mice (that were backcrossed to >99% C57BL/6 using speed congenics approaches) to impart IFN γ unresponsiveness either broadly in hematopoietic lineage cells or specifically in the CD8 α^+ /CD103 $^+$ DC subset, respectively (de Boer et al., 2003; Stranges et al., 2007). Using *Itgax-cre⁺Ifngr1^{ff}* mice, it was shown that IFN γ derived from TNF α -stimulated NK/NKT cells initiates the anti-*Listeria* response by triggering optimal IL-12 production by CD8 α^+ DCs. I also identified the importance of IFN γ responsiveness in neutrophils for control of *Listeria* infection

using *LysM-cre⁺Ifngr1^{ff}* mice. Adoptive transfer of WT neutrophils, not *Ifngr1^{-/-}* neutrophils can rescue *LysM-cre⁺Ifngr1^{ff}* mice which lack IFN γ responsiveness in neutrophils and a subset of macrophages.

CHAPTER 2

Experimental procedures

Generation of the *Ifngr*^{f/f} mice

Ifngr^{f/f} mice were generated by placing two loxP sites around the third and fourth exons that encode the extracellular domain of IFNGR1. A targeting vector, TNLOX1-3 (Radtke et al., 1999), was used to generate the *Ifngr* conditional targeting construct. A 1.3 kb fragment containing a part of the second intron, a 3.1 kb fragment containing the third and fourth exons, and a 4.4 kb fragment with an exogenous BamHI site containing the fifth and sixth exons of the murine *Ifngr* were cloned into the Not I, Asc I, and PmeI sites respectively, of the targeting vector.

The *Ifngr* targeting construct was linearized by SalI (NEB) digestion and electroporated into the B6/Blu embryonic stem cell line (Graubert et al., 1998). ES cells were subjected to 600 µg/ml of G-418 selection and single colonies were analyzed for homologous recombination by PCR using a 5' external primer (f3: 5'-ccagtgttgcctttggatctg-3', IDT) and a Neo primer (r3: 5'-gttgctaccgtgatattgctg-3', IDT) (Figure 1A). PCR positive clones were verified by Southern blot analysis using BamHI restriction digest with 5' and 3' external probe (Figure 1A and Figure S2A). One ES clone with the complete integration of the 3' region was further verified by PCR using each loxP specific primer set (f1: 5'-aaacagtaaaccagggtttgtac-3'; r1: 5'-cagcctctgaaattcaaattggc-3'; f2: 5'-gtgacgggagcacctgttac-3'; r2: 5'-gtaagtgcattcatctggccag-3',

IDT). Southern blot analysis using Neo probe demonstrated a single integration of the targeting vector into the endogenous *Ifngr1* locus (Figure 1A and data not shown).

The targeted ES clone was expanded and transiently transfected with pTurbo-Cre (ES cell core, Washington University) for removal of Neo resistance gene from the targeted allele of *Ifngr1*. Neo-sensitive clones were selectively picked and screened using 5' primers (5'-aaacagtaaaccagggtttgtac-3', IDT) and two 3' primers (5'-cagcctctgaaattcaaattggc-3', 5'-cgtggcactgtagatgtactgtcag-3', IDT) and were confirmed by Southern blot analyses using the 5' probe. Neo-deleted subclones were injected into B6.8 cell embryo using the laser-assisted microinjection technique (Poueymirou et al., 2007).

Mice

B6 (C57BL/6NTac) mice were obtained from Taconic. *Itgax-cre* (007567; C57BL/6J-Tg(*Itgax-cre*,-EGFP)4097Ach/J) mice (Stranges et al., 2007) and *Vav-icre* (008610; B6.Cg-Tg(*Vav1-cre*)A2Kio/J) mice (de Boer et al., 2003) were obtained from the Jackson Laboratory. To ensure pure B6 background, both *Itgax-cre* and *Vav-icre* mice were backcrossed onto the C57BL/6 background by speed congenic analysis (>99% purity, Rheumatic Diseases Core Center, Washington University) and then crossed to *Ifngr1^{ff}* mice. Mice were maintained in a specific pathogen-free facility in accordance with American Association for Laboratory Animal Science guidelines, and all protocols involving laboratory animals were approved by the Washington University Animal Studies Committee (School of Medicine, Washington University in St. Louis).

Infection

Listeria monocytogenes (stain: EGD) from frozen glycerol stocks at -80 °C was diluted into pyrogen-free saline. Mice were typically infected with a 10⁵ dose of *Listeria* i.p. for CFU measurement and with a 10⁶ dose of *Listeria* i.v. for intracellular cytokine staining. To measure bacterial burden, spleens and livers were harvested, homogenized in PBS containing 0.05% Triton X-100, and plated onto brain heart infusion media. Bacterial CFUs were determined after overnight incubation at 37 °C. In some experiments, small portions of spleens and livers were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E).

Antibody treatment

For *in vivo* depletion of NK/NKT cells, *Ifngr*^{ff} mice were treated with 200 µg anti-NK1.1 mAb (PK136, Biolegend) i.p. on days -2 and 0 (relative to *Listeria* infection). Memory phenotype CD8⁺ T cell depletion was achieved by injecting *Ifngr*^{ff} mice i.p. with 200 µg anti-CXCR3 mAb (CXCR3-173) on days -3 and 0. For IL-18 neutralization, 500 µg of anti-IL-18 mAb (SK113AE-4)(Lochner et al., 2002) was injected i.p. on day -1. For IL-1α/β/R neutralization, *Ifngr*^{ff} mice were injected i.p. with 250 µg each of anti-IL1α mAb (ALF-16)(Fuhlbrigge et al., 1988), anti-IL-1β mAb (B122)(Hogquist et al., 1991), and anti-IL-1R mAb (JAMA-147)(Rogers et al., 1992) on day -1. Dual TNFR1 and TNFR2 blockade was achieved by i.p. injection of a mixture containing 250 µg each of TNFR1 mAb (55R-170)(Sheehan et al., 1995) and TNFR2 mAb (TR75-54)(Sheehan

et al., 1995) on day -1. For IFN γ neutralization, 250 μ g of IFN γ -specific H22 mAb (Schreiber et al., 1985) was injected i.p. on day -1.

Flow cytometry

The following monoclonal antibodies were purchased from Biolegend as lineage markers: PerCP/Cy5.5 anti-CD3 ϵ (145-2C11), PE or APC anti-NK1.1 (PK136), FITC or PE/Cy7 anti-CD4 (GK1.5), PerCP/Cy5.5 or APC/Cy7 anti-CD8 α (53-6.7), FITC or PE anti-CD45R/B220 (RA3-6B2), APC anti-CD317/PDCA-1 (927), FITC or PerCP/Cy5.5 anti-F4/80 (BM8), PerCP/Cy5.5 or PE/Cy7 anti-CD11b (M1/70), PE anti-CD115 (AFS98), APC/Cy7 anti-Ly6G&C/Gr-1 (RB6-8C3), APC/Cy7 anti-Ly6C (HK1.4), PerCP/Cy5.5 anti-Ly6G (1A8), FITC or APC/Cy7 anti-CD11c (N418), PerCP/Cy5.5 anti-CD103 (2E7), APC anti-CD183/CXCR3 (CXCR3-173), FITC anti-CD62L (MEL-14), PE anti-CD44 (IM7), PE anti-CD31 (MEC13.3). PE anti-CD122 (TM- β 1) and PE anti-Siglec-F (E50-2440) were purchased from BD Biosciences. APC anti-DEC205 (205yekta) was purchased from eBioscience.

Single cell suspension from various tissues was prepared as following. Spleens and thymuses were dispersed into single cells by homogenizing between the frosted ends of the slides. Red blood cells were then lysed using red blood cell (RBC) lysing buffer (Sigma) prior to passing through an 80 μ m strainer. Livers were perfused with HBSS via portal vein, and pressed through a stainless steel mesh. Cell suspension was centrifuged at 50 x g for 30 sec to remove hepatocytes. Supernatant was overlaid onto histopaque-1083 (Sigma) and centrifuged at 600 x g for 25 min at 25 $^{\circ}$ C. The visible interface was

collected, washed, and passed through 80 μ m strainer. Blood was collected into PBS containing 5mM EDTA and lysed using RBC lysing buffer (Sigma). To harvest peritoneal cavity resident cells, peritoneal cavities were lavaged with 10 ml PBS.

For staining for IFNGR1, cells were blocked with Fc Block (BD Biosciences) in FACS buffer (PBS/2% fetal calf serum/0.1% sodium azide) on ice for 15 min. Cells were stained with indicated lineage markers and biotin anti-IFNGR1 (GR20, BD Biosciences) on ice for 1 hour (Figure 1). Cells were washed and stained with PE or APC-SA (Biolegend) on ice for 30 min. Dead cells were excluded by PO-PROTM-1 (Invitrogen) counterstaining. For intracellular pSTAT1 staining, cells were stained with indicated lineage markers on ice for 1 hr following Fc Block, and washed twice. After incubation with 1,000 units/ml IFN γ at 37 $^{\circ}$ C for 15 min, cells were fixed in 2% paraformaldehyde/PBS at 37 $^{\circ}$ C for 10 min and permeabilized with 90% methanol/PBS on ice for 1 hr. Cells were subsequently stained with Alexa647 anti-pSTAT1 (4a, BD Biosciences) at RT for 1 hr. For intracellular cytokine staining, cells were stained with lineage markers without *in vitro* re-stimulation or incubation with intracellular transport blockers. To label dead cells, Fixable Viability Dye eFluor[®] 450 (eBioscience) was used prior to fixation and permeabilization procedures with BD Cytofix/CytopermTM (BD biosciences). Cells were subsequently stained with PE anti-IL-12p40 (C15.6, Biolegend), PE/Cy7 anti-IFN γ (XMG1.2, Biolegend), PE/Cy7 anti-TNF α (MP6-XT22, Biolegend), and goat anti-NOS2 (Santa Cruz) followed by PerCP/Cy5.5 donkey anti-goat IgG (Santa Cruz).

Immunohistochemistry

Alexa488 anti-B220 (RA3-6B2), biotin anti-CD3 ϵ (145-2C11), and biotin anti-CD11b (M1/70) were purchased from BD Biosciences. Alexa555-SA, Alexa555 goat anti-Rat IgG (H+L highly Cross-Adsorbed), and Alexa647 goat anti-rabbit IgG (H+L highly Cross-Adsorbed) were purchased from Invitrogen. *Listeria* O antiserum Poly Type 1 & 4 was purchased from BD Diagnostics. Alexa647 anti-CD11c (N418) was purchased from eBioscience. Biotin anti-Siglec-1(MOMA-1) was purchased from BMA Biomedicals. Rat anti-MARCO was purchased from Serotec.

Fresh spleens were embedded in Tissue-Tec OCT (Fisher) and 7 μ m sections were cut. Frozen sections were brought to RT and fixed in acetone at 4 °C for 5 min. All slides were blocked with CAS Block (Invitrogen) and stained with indicated antibodies diluted in CAS Block. Four-color epifluorescence microscopy was performed with an Olympus BX51 microscope equipped with a SPOT RT CCD camera (Diagnostic Instruments). Monochrome images were acquired through DAPI/FITC/TRITC/Cy5 fluorescence filters (Chroma), pseudo-colored with SPOT RT camera software, and merged with Adobe Photoshop.

Cytometric bead array (CBA)

Serum levels of IL-6, TNF α , IFN γ , and MCP-1 were accessed by using CBA Mouse Inflammation Kit (BD Biosciences) according to the manufacturer's instructions. CBA data was analyzed with CELLQuest™ software and the CBA analysis software package (BD Biosciences).

Adoptive transfer of neutrophils

The femur and the tibia from both fore and hind legs were removed from mice, and the tip of each extremity was cut off. Bone marrow was flushed out through the bone using HBSS solution with a 23G syringe. After dispersing cell clumps using a 20G syringe, the cell suspension was centrifuged (400g, 10 min, 4°C) and lysed using RBC lysing buffer (Sigma). The cells were re-suspended in PBS, applied onto a two-layer Percoll gradient of 80% and 62% Percoll (Amersham Pharmacia Biotech) in PBS (100% Percoll contains nine parts Percoll and one part 10× PBS), and centrifuged (1000 g, 30 min, room temperature) with the centrifuge brake turned off. The fraction from the 62%/80% interface was harvested into PBS/0.5%FCS/2mM EDTA. This fraction was further purified using Ly6G⁺ MACS purification kit (Miltenyi). The isolated cells were analyzed by cyto-spin centrifugation followed by Wright-Giemsa staining (Merck). I obtained 1×10^7 cells per mouse on average. The 3×10^6 neutrophils were adoptively transferred into recipient mice intravenously as shown in Figure 35A.

Statistical analysis

The statistical analysis for differences in CFUs was performed by the Mann-Whitney test using Prism software (GraphPad Software). In the figures, each circle (or triangles) represents an individual mouse, and the bar indicates the mean value of the group. P values less than or equal to 0.05 were considered to be significant. Error bars indicate standard error of the mean (SEM).

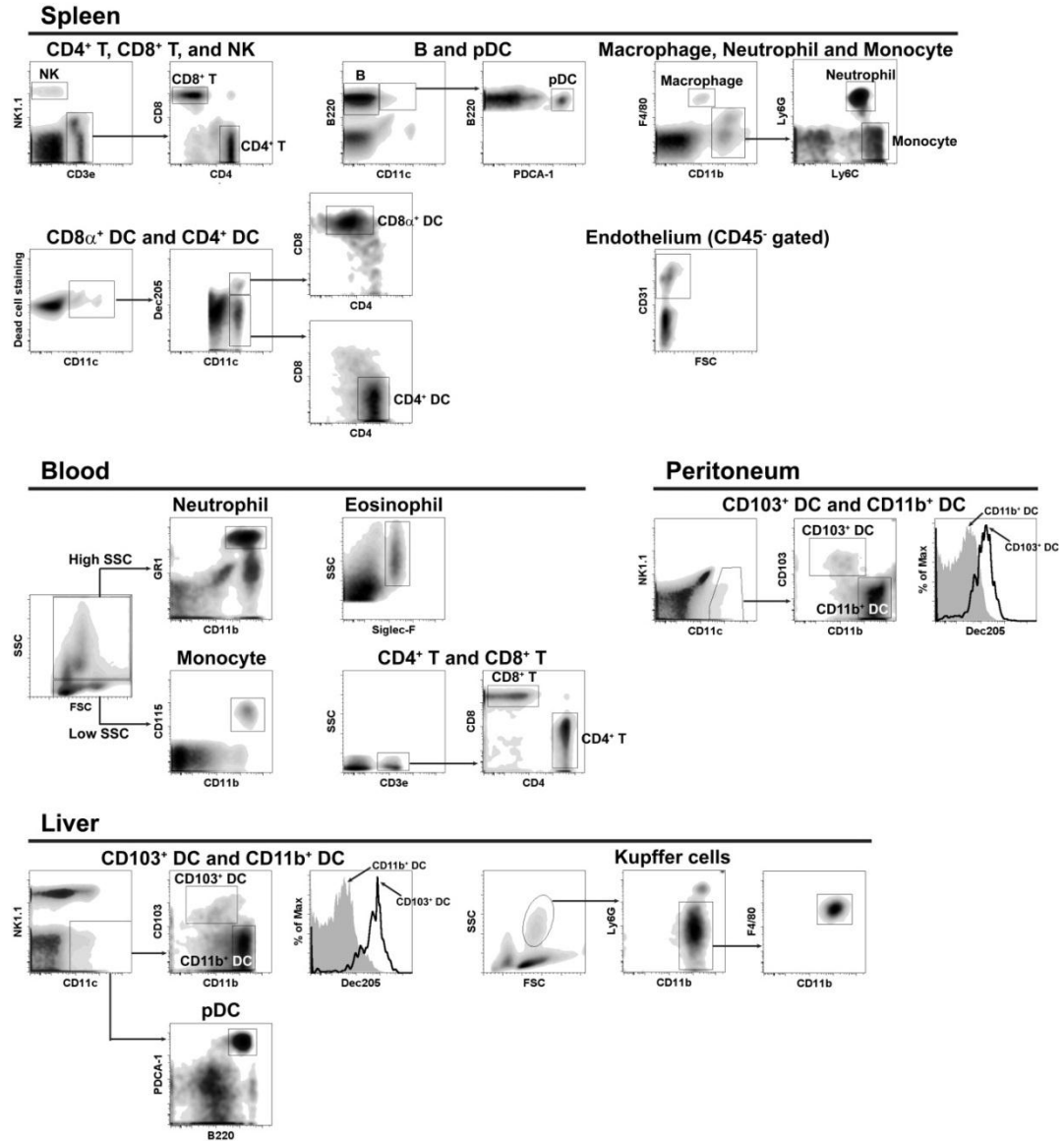


Figure 1. Gating strategy and markers for delineation by flow cytometry

Cells from spleen were labeled with antibodies against lineage markers to detect the following populations; CD3 ϵ ⁺CD4⁺CD8 α ⁻ T cell, CD3 ϵ ⁺CD4⁻CD8 α ⁺ T cell, CD3 ϵ ⁻NK1.1⁺ NK cell, CD3 ϵ ^{int}NK1.1^{int} NKT cell, B220⁺ B cell, CD11c⁺B220⁺PDCA-1⁺ pDC, CD11b^{int}F4/80⁺ macrophage, CD11b⁺Ly6C⁺Ly6G⁻ monocyte, CD11b⁺Ly6C^{int}Ly6G⁺ neutrophil, CD11c⁺Dec205⁺CD8 α ⁺ DC, CD11c⁺Dec205⁻CD4⁺CD11b⁺ DC, and CD45⁻

CD31⁺ endothelial cells. Cells from blood were labeled with antibodies against lineage markers to detect the following populations; SSC^{hi}CD11b⁺GR1⁺ neutrophil, SSC^{hi}SiglecF⁺ eosinophil, SSC^{lo}CD11b⁺CD115⁺ monocyte, SSC^{lo}CD3ε⁺CD4⁺CD8α⁻ T cell, and SSC^{lo}CD3ε⁺CD4⁻CD8α⁺ T cell. Cells from peritoneal cavity and liver were labeled with antibodies against lineage markers to detect the following populations; CD11c⁺NK1.1⁻CD103⁺Dec205⁺CD11b⁻ DC, CD11c⁺NK1.1⁻CD103⁻Dec205⁻CD11b⁺ DC, SSC^{hi}CD11b⁺Ly6G⁻F4/80⁺ Kupffer cell, and CD11c⁺NK1.1⁻B220⁺PDCA-1⁺ pDC.

CHAPTER 3

Generation and characterization of *IfngrI^{ff}* mice

We generated a conditional knockout allele of *Ifngr1* in pure C57BL/6 strain ES cells by placing two loxP sites surrounding the third and fourth exons that encode the extracellular domain of IFNGR1. Deletion of this gene region induces a frame shift mutation after exon 2, leaving only a minimal portion of IFNGR1 that would not bind IFN γ (Figure 2). Southern blotting and PCR analyses for both loxP sites showed a correct genotype of *IfngrI^{ff}* (Figure 3A and B). Flow cytometric analysis of spleen, peripheral blood, and thymus revealed that *IfngrI^{ff}* mice were indistinguishable from normal C57BL/6 mice on the basis of surface expression of IFNGR1 in hematopoietic cells (Figure 4). *IfngrI^{ff}* mice did not display any developmental abnormalities and showed no problems reproducing.

In addition, *IfngrI^{-/-}* ES cell subclones in which the entire floxed region was deleted were also obtained (Figure 5). The *IfngrI^{-/-}* allele was germline-transmitted (Figure 6) giving rise to mice named *IfngrI_{WU}^{-/-}* to distinguish them from *IfngrI^{-/-}* mice previously generated on a 129/SvEv background by M. Aguet (Huang et al., 1993). Cells from *IfngrI_{WU}^{-/-}* mice neither expressed IFNGR1 nor displayed induction of phosphorylated STAT1 (pSTAT1) after *in vitro* treatment with IFN γ (Figure 4 and 7). However, cells from *IfngrI_{WU}^{-/-}* mice were fully responsive to treatment with Type I Interferons, specifically IFN α 5 and IFN β .

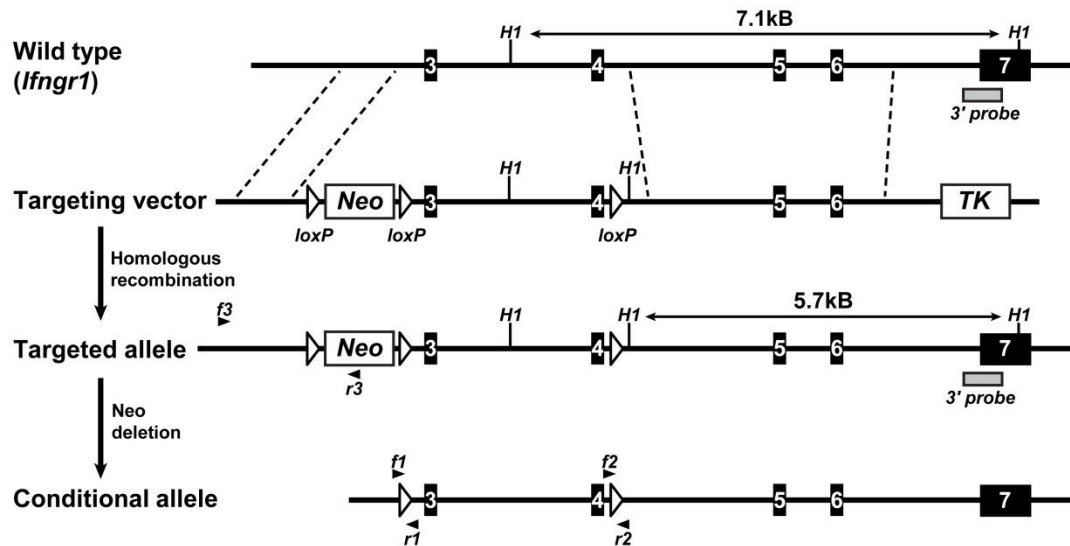


Figure 2. Targeting strategy for *Ifngr1*^{f/f} mice

Only five (black vertical bar or box) of seven total exons are shown for simplicity. H1, BamH1; Neo, neomycin resistance gene; TK, herpes simplex virus thymidine kinase gene; open triangle, loxP site. Open gray box indicates 3' external southern probe to verify 3' homologous recombination. Small arrowheads indicate pairs of primers used in PCRs to confirm either 5' homologous recombination (f3/r3) or insertion of loxP sites (f1/r1 and f2/r2).

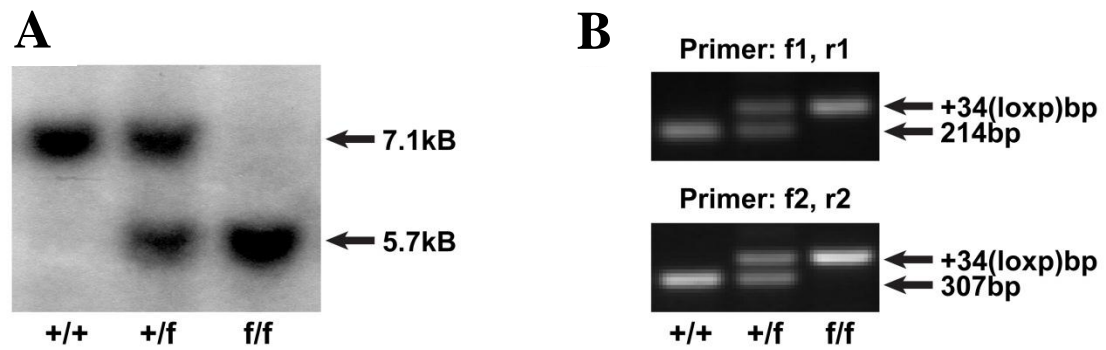


Figure 3. Germline transmission of *Ifngr1*^{f/f} mice

(A) Confirmation of germline transmission by examining 3' side of targeted allele by southern blotting. The wild type (+) allele gives the 7.1kb band and the targeted (f) allele gives the 5.7kb band (Figure 2).

(B) Verification of correct insertion of both 34 bp-long loxP sites by PCR reactions. Both reactions show 34 bp increased band size in the targeted allele compared to the wild type allele.

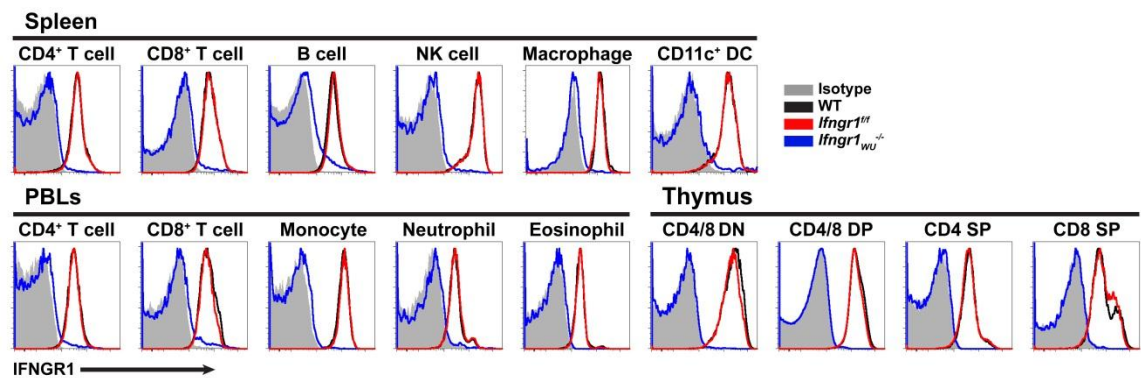


Figure 4. The normal expression of IFNGR1 in *Ifngr1^{fl/fl}* mice

Expression of IFNGR1 was assessed by FACS analyzing spleen, peripheral blood leukocytes (PBLs), and thymus from wild type, *Ifngr1^{fl/fl}*, and *Ifngr1^{WU-/-}* mice. Data are representative of three separate experiments.

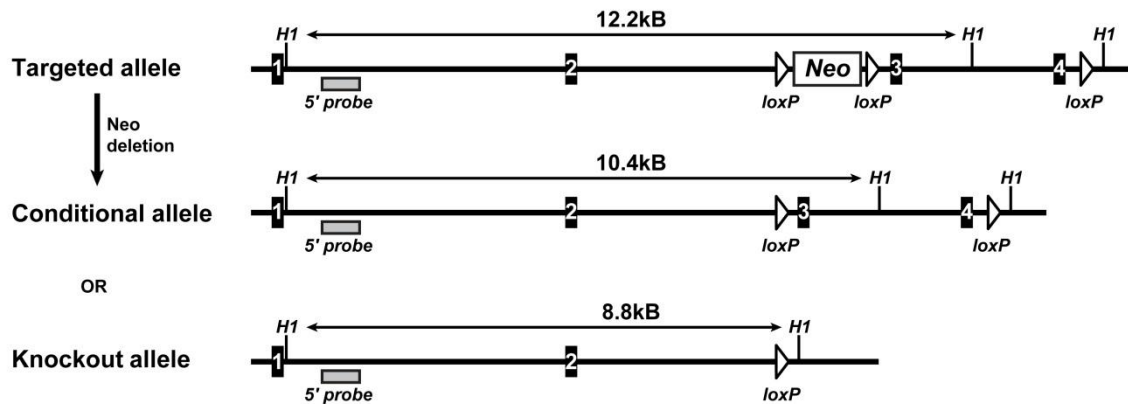


Figure 5. Screening strategy for *Ifngr1^{wu}* mice

Only four (black vertical bar or box) of 7 total exons are shown for simplicity. Open gray box indicates 5' external southern probe to verify both 5' homologous recombination and cre-mediated deletion.

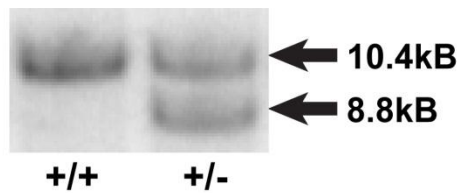


Figure 6. Germline transmission of *Ifngr1*_{WU}^{-/-} mice

Confirmation of germline transmission by examining 5' side of targeted allele by southern blotting. The wild type (+) allele gives the 10.4kb band and the knockout (-) allele gives the 8.8kb band.

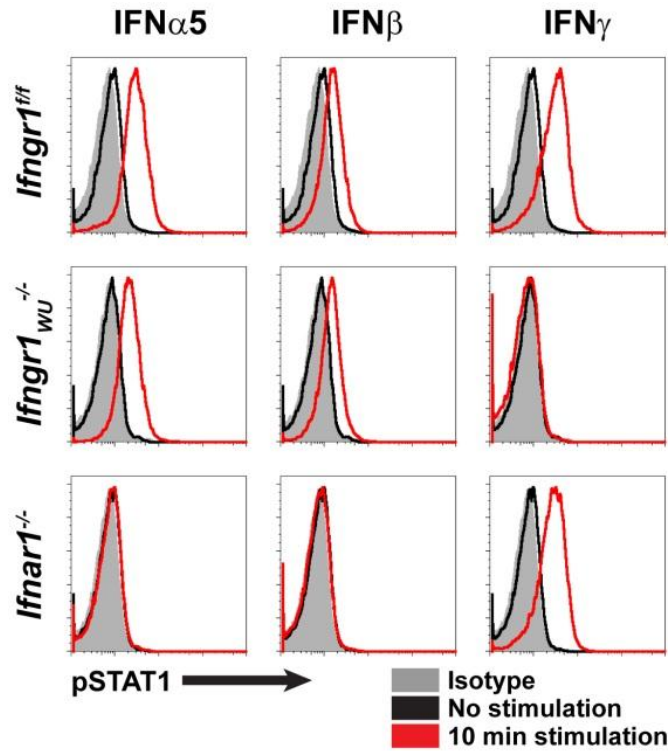


Figure 7. *Ifngr1*^{WU/-} mice show no phosphorylation of STAT1 after *in vitro* IFN γ stimulation

Measurement of phosphorylated STAT1 in *Ifngr1*^{WU/-} mice after *in vitro* IFN γ treatment. Spleens were harvested from *Ifngr1*^{fl/fl}, *Ifngr1*^{WU/-}, and *Ifnar1*^{-/-}, and treated with IFN α 5 (10,000 U/ml), IFN β (10,000 U/ml), and IFN γ (1,000 U/ml) for 10 minutes at 37°C *in vitro*. After stimulation, samples were fixed, permeablized, and stained with anti-phospho STAT1 antibodies.

CHAPTER 4

IFN γ responsiveness in hematopoietic cells is required to control *Listeria* infection

***Vav-icre*⁺*IfngrI*^{ff} mice show the complete deletion of *IfngrI* in hematopoietic cells**

To selectively delete *IfngrI* in hematopoietic cells, *IfngrI*^{ff} mice were bred to *Vav-icre* mice. *Vav* promoter elements induce ubiquitous expression of transgenes in all hematopoietic cells (de Boer et al., 2003). CD8 α ⁺ DCs, CD4⁺ DCs, macrophages, NK cells, CD4⁺ T cells, CD8⁺ T cells, B cells, monocytes, and neutrophils from spleen of *Vav-icre*⁺*IfngrI*^{ff} mice neither expressed IFNGR1 on their surface nor phosphorylated STAT1 after *in vitro* IFN γ stimulation (Figure 8 and 9). In contrast, CD31⁺CD45⁻ endothelial cells expressed IFNGR1, indicating that IFNGR1 expression in the non-hematopoietic compartment was not affected in *Vav-icre*⁺*IfngrI*^{ff} mice.

***Vav-icre*⁺*IfngrI*^{ff} mice succumb to *Listeria* infection**

Next, I challenged *Vav-icre*⁺*IfngrI*^{ff} mice with a 10⁵ dose of *Listeria* i.p., and found that both *Vav-icre*⁺*IfngrI*^{ff} and *IfngrI*_{WU}^{-/-} mice succumbed to infection by day 5, whereas *IfngrI*^{ff} mice resolved the infection (Figure 10A). Bacterial counts in spleens from *Vav-icre*⁺*IfngrI*^{ff} and *IfngrI*_{WU}^{-/-} mice contained 10-fold more bacteria at day 1 and 100- to 1000-fold more *Listeria* at day 3 than control *IfngrI*^{ff} mice (Figure 10B). Similar results were also observed for liver colony counts. There were no significant differences in bacterial burdens between *Vav-icre*⁺*IfngrI*^{ff} and *IfngrI*_{WU}^{-/-} mice. Increased bacterial burdens in *Vav-icre*⁺*IfngrI*^{ff} mice were evidenced by significantly increased numbers of

Listeria foci in both the spleen and liver compared to *IfngrI^{ff}* mice (Figure 11). Thus, our *Vav-icre⁺IfngrI^{ff}* mice functionally recapitulate the defect previously noted in *IfngrI^{-/-}* BM chimeras (Yap and Sher, 1999) with respect to their requirement for IFN γ responsiveness in the hematopoietic compartment.

It was measured serum cytokine/chemokine from blood in *IfngrI^{ff}* and *Vav-icre⁺IfngrI^{ff}* mice at day 1 and 3 after infection using a cytometric bead array (Fig. 12). Increased production of the major inflammatory cytokines such as IFN γ and IL-6 in the early stage of infection was observed, which indicates increased bacterial burden in *Vav-icre⁺IfngrI^{ff}* mice *in vivo* coincides with increased cytokine production.

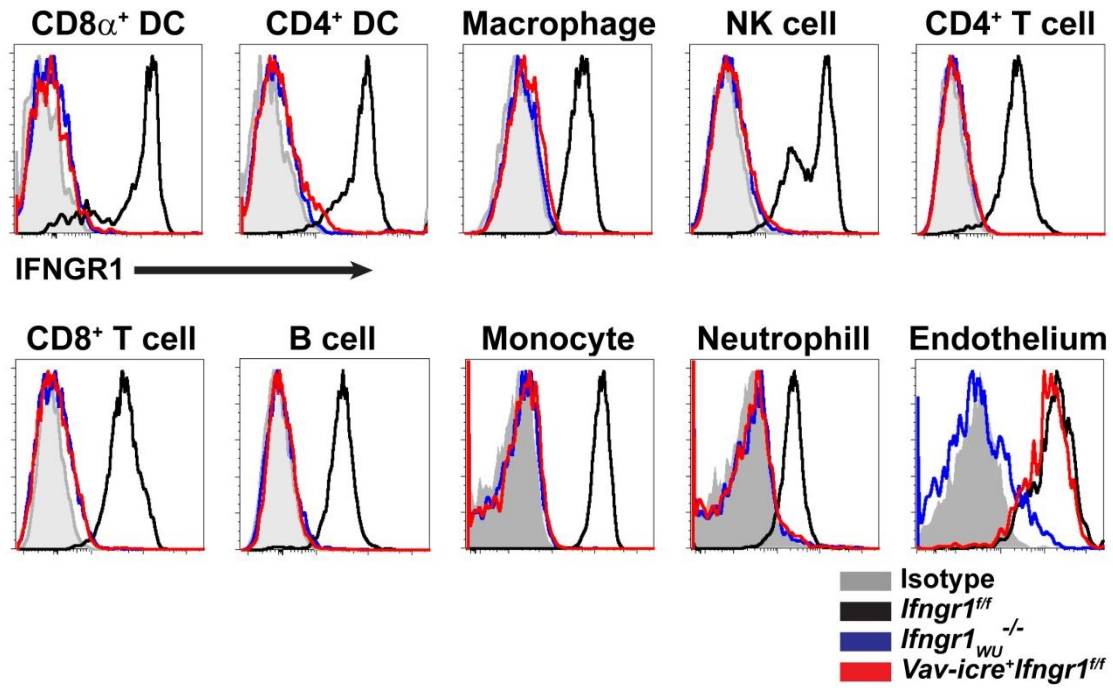


Figure 8. *Vav-icre⁺Ifngr1^{f/f}* has no IFNGR1 in hematopoietic compartment

IFNGR1 expression on splenocytes was measured using flow cytometry in *Vav-icre⁺Ifngr1^{f/f}* mice. The spleens and PBLs from *Ifngr1^{f/f}*, *Vav-icre⁺Ifngr1^{f/f}*, and *Ifngr1^{wU}-/-* were harvested and stained with either biotinylated anti-IFNGR1 mAb or Isotype Ab with various lineage markers (Figure 1). Data are representative of two separate experiments

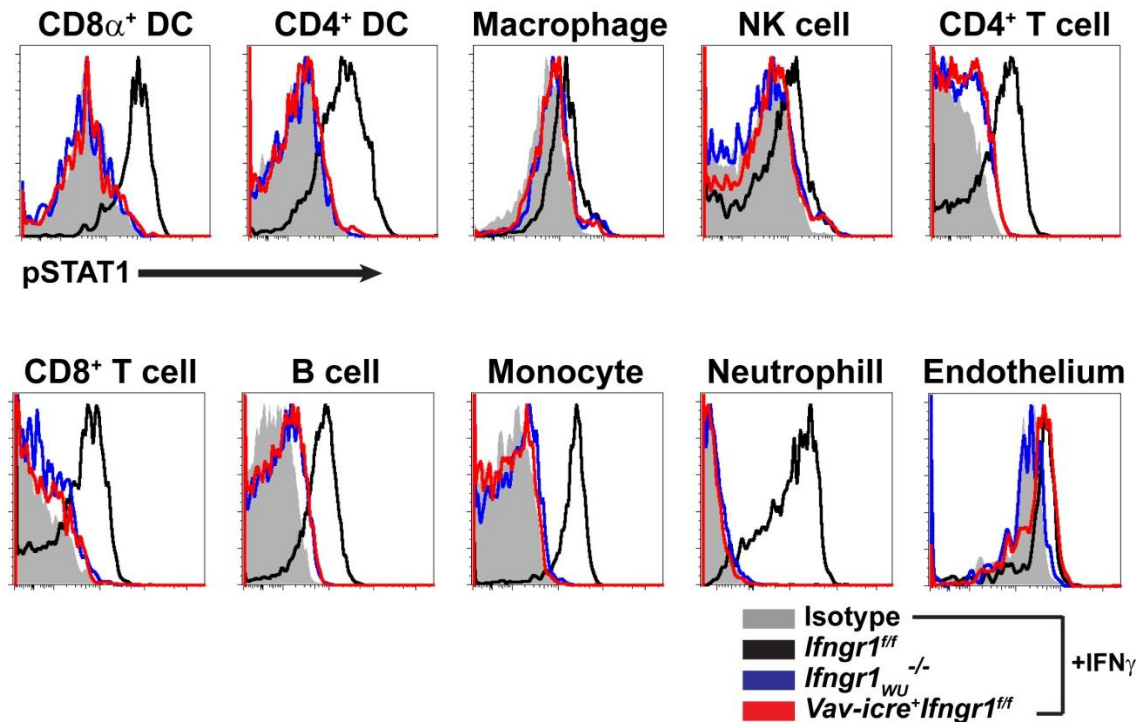


Figure 9. *Vav-icre*⁺*Ifngr1*^{ff} has no phosphorylation of STAT1 in hematopoietic compartment after *in vitro* IFN γ stimulation

Measurement of phosphorylated STAT1 in *Vav-icre*⁺*Ifngr1*^{ff} mice after *in vitro* IFN treatment. Both spleen and PBLs were harvested from *Ifngr1*^{ff}, *Vav-icre*⁺*Ifngr1*^{ff}, and *Ifngr1*^{wu/-}. They were first stained with lineage markers and treated with IFN γ (1,000 U/ml) for 15 minutes at 37°C *in vitro*. After stimulation, samples were fixed, permeabilized, and stained with anti-pSTAT1 antibody. Data are representative of two separate experiments.

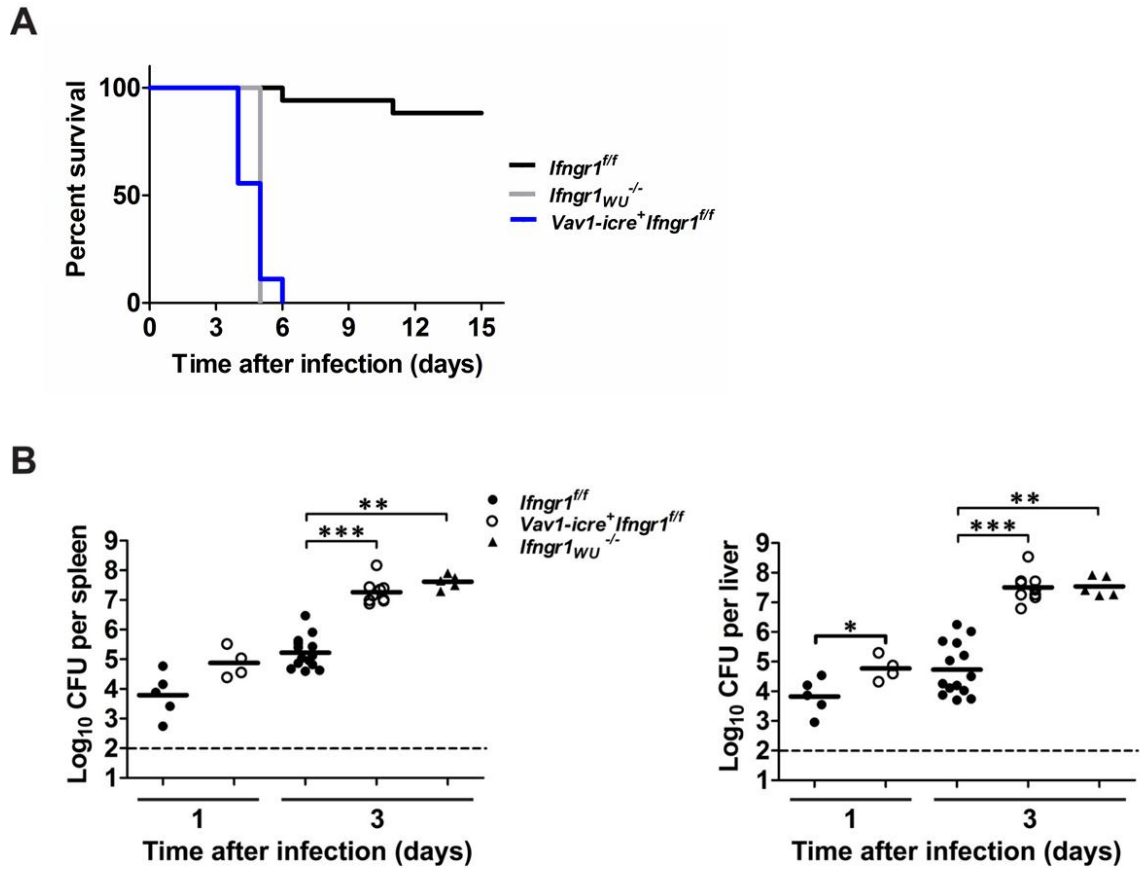


Figure 10. *Vav-icre⁺ Ifngr1^{f/f}* mice are susceptible to *Listeria* infection

(A) Survival of mice which were i.p. infected with 10^5 *Listeria*.

(B) *Listeria* CFUs per spleen and liver of *Ifngr1^{f/f}*, *Vav-icre⁺ Ifngr1^{f/f}*, and *Ifngr1^{WU-/-}* mice which were i.p. infected with 10^5 *Listeria*. Each symbol represents an individual mouse.

Lines represent the mean Log₁₀CFU. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. Data are a combination of at least two separate experiments.

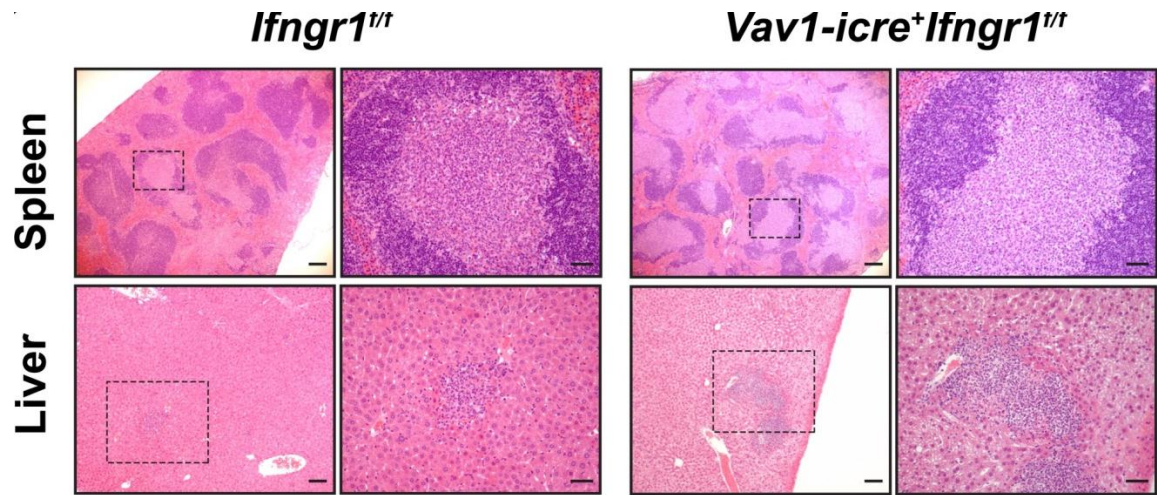


Figure 11. *Vav-icre⁺Ifngr1^{f/f}* mice have increased histological lesions in spleen and liver

H&E staining of infected spleens and livers from mice 3 days after infection (10^5 *Listeria* i.p.). Right panels show magnified regions (dashed boxes in left panels). Scale bar for spleen represents 200um in low-power images and 50um in high-power images; scale bar for liver represents 100um in low-power images and 50um in high-power images.

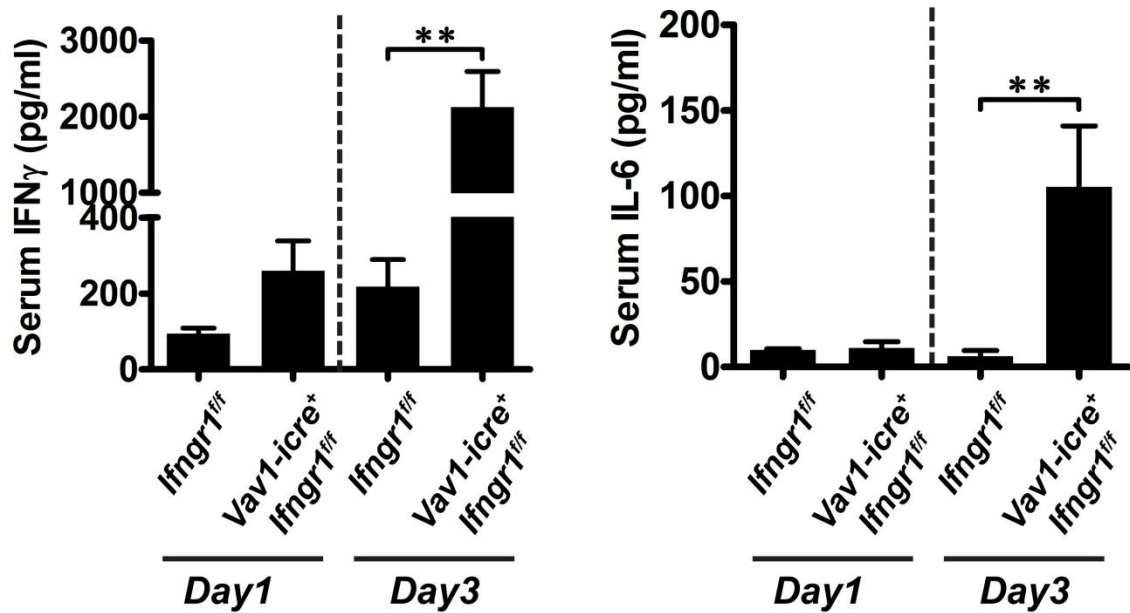


Figure 12. *Vav-icre⁺Ifngr1^{ff}* mice show increased production of early pro-inflammatory cytokines, IFN γ and IL-6

Infected *Ifngr1^{ff}* and *Vav-icre⁺Ifngr1^{ff}* were bled at indicated time points after infection, and serum was analyzed for IFN γ and IL-6 concentration using cytometric bead array.

Data represent averages of serum concentration of cytokines from at least two separated experiments ($n \geq 4$ at each time point).

CHAPTER 5

IFN γ responsiveness in CD8 α^+ /CD103 $^+$ dendritic cells is required for optimal control of *Listeria* infection

***Itgax-cre⁺Ifngr1^{ff}* mice show selective IFN γ unresponsiveness in CD8 α^+ /CD103 $^+$ dendritic cells**

To explore whether IFN γ responsiveness in DCs affects *Listeria* susceptibility in intact animals, *Ifngr1^{ff}* mice were bred with *Itgax-cre* mice to produce DC-specific deletion of *Ifngr1* (Stranges et al., 2007). A specific strain of *Itgax-cre* mice was selected because they had been generated directly on a C57BL/6 background and because our previous experience with these mice showed that they were efficient in directing deletion of floxed genes in dendritic cells and in the CD8 α^+ /CD103 $^+$ DC compartment particularly (Diamond et al., 2011). These *Itgax-cre* mice express cre recombinase under the control of the mouse integrin alpha X (CD11c) promoter which directs bicistronic cre and EGFP protein expression to DCs. Although expression of CD11c occurs in NK cells, monocytes, macrophages, neutrophils, and some B cells in addition to DCs, *Itgax-cre⁺Ifngr1^{ff}* mice showed significant reductions in IFNGR1 expression primarily in CD8 α^+ DCs, with partial reductions occurring in splenic CD4 $^+$ DCs and macrophages (Figure 13). The extent of *Ifngr1* deletion correlated well with the level of simultaneous EGFP expression (Figure 19). Having substantially diminished IFNGR1 expression in CD8 α^+ DCs, I measured the level of pSTAT1 and CD40 in these cells as functional readouts after *in vitro* IFN γ treatment. In *Itgax-cre⁺Ifngr1^{ff}* mice, CD8 α^+ DCs showed significant

inhibition of STAT1 phosphorylation and CD40 upregulation by IFN γ (Figure 14 and 15). In contrast, CD4⁺ DCs, macrophages, and the other hematopoietic cells showed relatively normal responses.

Splenic CD8 α ⁺ DCs and peripheral CD103⁺ DCs are thought to be functionally and developmentally related, as both subsets are efficient at cross-presentation of cell-associated antigens and *Batf3*^{-/-} mice lack both CD8 α ⁺ and peripheral CD103⁺ DCs, whereas other cell lineages remain intact (Bedoui et al., 2009; Hildner et al., 2008). As CD103⁺ DCs exist in all peripheral tissues, the deletion of *Ifngr1* in peritoneal and hepatic CD103⁺ DCs was analyzed (Ginhoux et al., 2009). It was not surprising to find that CD103⁺ DCs in the peritoneal cavity and liver exhibited an almost complete deletion of *Ifngr1* in *Itgax-cre*⁺*Ifngr1*^{ff} mice, whereas myeloid CD11b⁺ DCs from these tissues did not (Figure 16). No other cell populations in these mice were found to exhibit defect in either IFNGR1 expression or IFN γ responsiveness. Thus the *Itgax-cre*⁺*Ifngr1*^{ff} mice display a highly specific IFN γ unresponsiveness within the CD8 α ⁺/CD103⁺ DC compartment.

***Itgax-cre*⁺*Ifngr1*^{ff} mice show increased susceptibility to *Listeria* infection**

Both *Ifngr1*^{ff} and *Itgax-cre*⁺*Ifngr1*^{ff} were next challenged with a sublethal *Listeria* dose and found no difference in mortality between *Ifngr1*^{ff} and *Itgax-cre*⁺*Ifngr1*^{ff} mice (Figure 17A). However, *Itgax-cre*⁺*Ifngr1*^{ff} mice exhibited significantly increased *Listeria* burdens in both the spleen and liver during first 7 days of the infection, and showed a 10- to 100-fold increase in colony forming units (CFUs) over *Ifngr1*^{ff} mice

at day 3 (Figure 17B). Increased bacterial colonization was more prominent in the liver than the spleen in *Itgax-cre⁺Ifngr1^{ff}* mice, due to the fact that resolution of *Listeria* infection in the spleen began after day 3, but did not occur in the liver until day 7. Histologically, increased bacterial burdens in *Itgax-cre⁺Ifngr1^{ff}* mice were reflected in significantly increased numbers and sizes of *Listeria* foci in both the spleen and liver compared to *Ifngr1^{ff}* mice (Figure 18). Despite the defects in the initial control of *Listeria* infection, *Itgax-cre⁺Ifngr1^{ff}* mice were still able to develop sterilizing immunity and resolve *Listeria* infection by day 15 (Figure 17B).

Two sets of data revealed that the increased susceptibility to *Listeria* infection in *Itgax-cre⁺Ifngr1^{ff}* mice was due solely to IFN γ unresponsiveness in the CD8 α^+ /CD103 $^+$ DCs. First, *Listeria* infection did not up-regulate expression of the transgenic cre-EGFP bicistronic construct (derived from the *Itgax-cre* mice) in other cell types thus ruling out a temporally delayed deletion of IFNGR1 in them. Specifically, strong expression of EGFP was detected in CD8 α^+ DCs, less in CD4 $^+$ DCs, and not at all in other cell types before or after *Listeria* infection (Figure 19). Second, previous histological studies showed that *Listeria* is initially trapped by marginal zone macrophages (MZM) and metallophilic MZM (MMM) in the splenic marginal zone, where these cells have direct access to bacteria entering the spleen from the circulation (Aichele et al., 2003; Aoshi et al., 2009). Thus, deletion of IFNGR1 in these cells could potentially favor *Listeria* expansion during the initial stages of infection. Because of technical difficulties in detecting MZM and MMM by flow cytometry, tissue frozen sections were co-stained for MARCO1, MOMA-1, and CD11c, then analyzed sections by fluorescence microscopy (Figure 20). Neither

MARCO1⁺ MZM nor MOMA-1⁺ MMM showed CD11c expression with or without *Listeria* infection, which ruled out alterations in IFN γ responsiveness. Thus, increased susceptibility to *Listeria* infection in *Itgax-cre⁺Ifngr1^{ff}* was solely due to IFN γ unresponsiveness in the CD8 α ⁺/CD103⁺ DC population.

IFN γ unresponsiveness in CD8 α ⁺ DCs affects neither the transport of *Listeria* nor the formation of granulomatous lesions in PALS

CD8 α ⁺ DCs are responsible for the transport of *Listeria* from the splenic marginal zone to the periarteriolar lymphoid sheath (PALS) (Edelson et al., 2011; Neuenhahn et al., 2006). They are also involved in the subsequent formation of an early granulomatous lesion in the PALS that contains granulocytes, NK cells, and monocytes (Kang et al., 2008). Thus, it was assessed whether a deficit in IFN γ responsiveness by CD8 α ⁺ DCs affects these events and results in increased susceptibility to *Listeria* infection in *Itgax-cre⁺Ifngr1^{ff}* mice. To address this issue, I examined the location of *Listeria* and the formation of granulomatous lesions by immunohistochemistry at 18 hours following *Listeria* infection (Figure 21). In both *Ifngr1^{ff}* and *Itgax-cre⁺Ifngr1^{ff}* mice, CD11b⁺ myeloid cells were clustered in the PALS as delineated by CD3 ϵ (T cells) and B220 (B cells) staining. I also found extensive growth of *Listeria* which co-localized with CD11b⁺ myeloid cells in PALS. These results suggest that both the normal migration of *Listeria* infected CD8 α ⁺ DCs and the subsequent clustering of innate cells occurred regardless of their ability to respond to IFN γ .

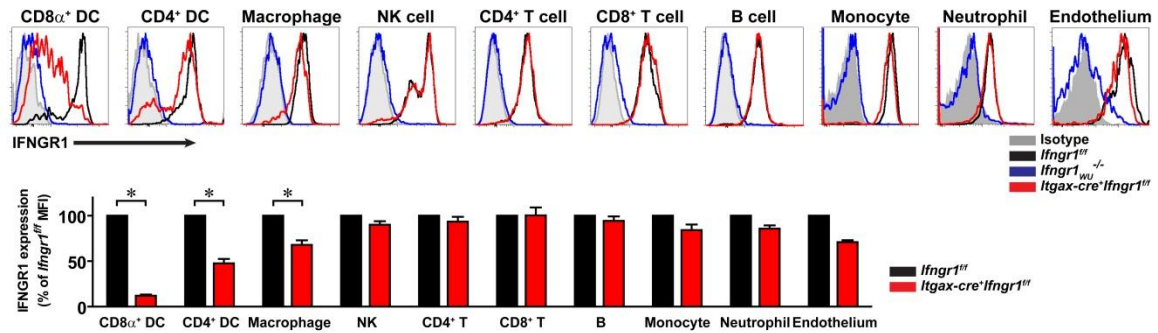


Figure 13. *Itgax-cre⁺Ifngr1^{fl/fl}* has no IFNGR1 in splenic CD8α⁺ DCs

Flow cytometry analysis to show no IFNGR1 expression in splenic CD8α⁺ DCs in *Itgax-cre⁺Ifngr1^{fl/fl}* mice. Data are representative of four separate experiments. IFNGR1 levels in the indicated cellular subsets in *Itgax-cre⁺Ifngr1^{fl/fl}* mice compared with *Ifngr1^{fl/fl}* mice were summarized in bar graph (expressed as a percentage of the mean fluorescence intensity)

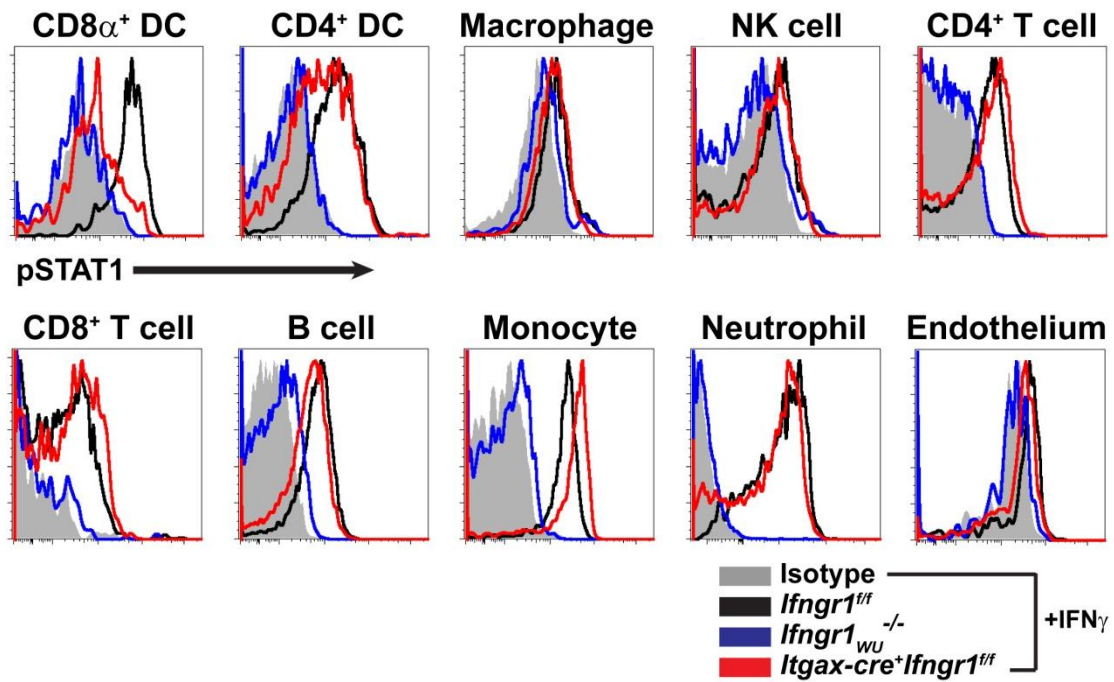


Figure 14. *Itgax-cre*⁺*Ifngr1*^{f/f} has no phosphorylation of STAT1 in CD8 α^+ DCs after *in vitro* IFN γ stimulation

Measurement of phosphorylated STAT1 in splenic DCs in *Itgax-cre*⁺*Ifngr1*^{f/f} mice after *in vitro* IFN γ treatment (1,000 U/ml) for 15 minutes at 37°C. Data are representative of two separate experiments.

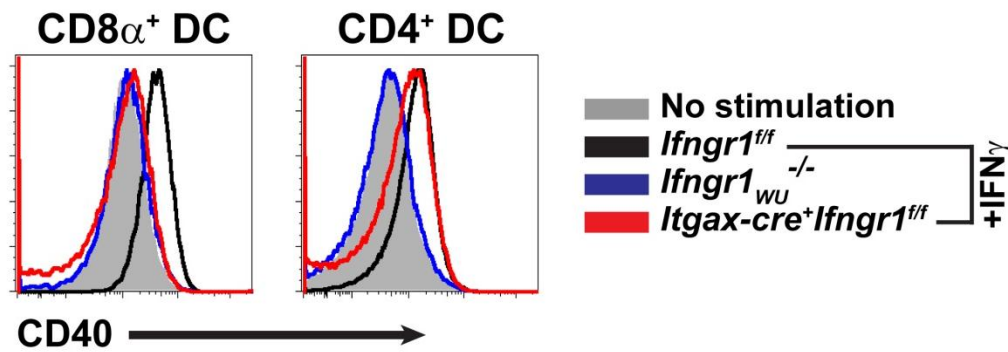


Figure 15. *Itgax-cre⁺Ifngr1^{ff}* has no up-regulation of CD40 in CD8 α^+ DCs after *in vitro* IFN γ stimulation

Up-regulation of CD40 in splenic DCs in *Itgax-cre⁺Ifngr1^{ff}* mice after *in vitro* IFN γ treatment. Splenic CD11c $^+$ cells were positively enriched by MACS purification from *Ifngr1^{ff}*, *Itgax-cre⁺Ifngr1^{ff}*, and *Ifngr1^{wu}*^{-/-}. They were treated with IFN γ (500 U/ml) for 18 hours at 37°C *in vitro* and stained with anti-CD40 antibodies and lineage markers.

Data are representative of two separate experiments.

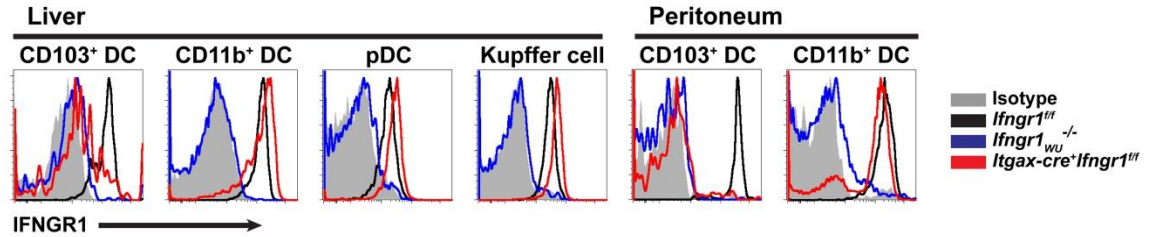


Figure 16. *Itgax-cre⁺Ifngr1^{fl/fl}* has no IFNGR1 in peripheral CD103⁺ DCs

FACS analysis to confirm no IFNGR1 expression in peripheral CD103⁺ DCs in *Itgax-cre⁺Ifngr1^{fl/fl}* mice. The peritoneal cavity and liver from *Ifngr1^{fl/fl}*, *Itgax-cre⁺Ifngr1^{fl/fl}*, and *Ifngr1^{wu}^{-/-}* were harvested and stained with either biotinylated anti-IFNGR1 mAbs or Isotype Ab with various lineage markers (Figure S5). Data are representative of two separate experiments.

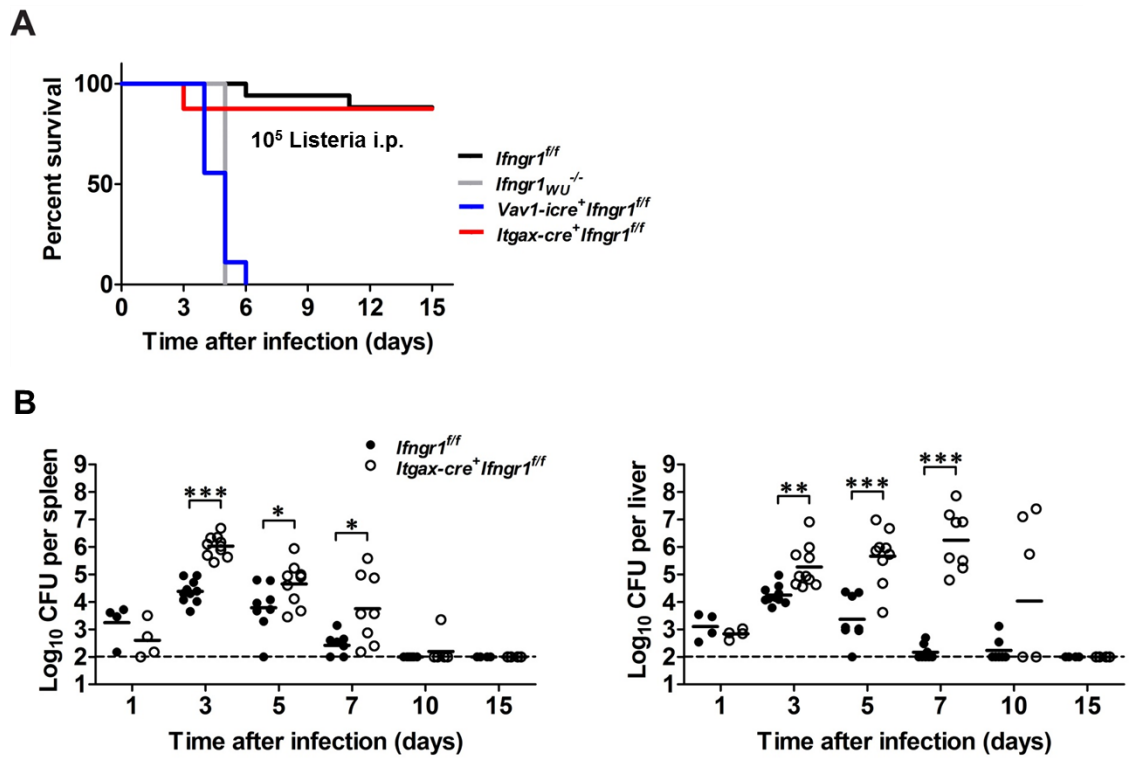


Figure 17. *Itgax-cre⁺Ifngr1^{f/f}* mice are susceptible to *Listeria* infection

(A) Survival of mice which were i.p. infected with 10⁵ *Listeria*.

(B) *Listeria* CFUs per spleen and liver of *Ifngr1^{f/f}*, *Itgax-cre⁺Ifngr1^{f/f}*, and *Ifngr1^{wU-/-}* mice which were i.p. infected with 10⁵ *Listeria*. Each symbol represents an individual mouse. Lines represent the mean Log₁₀CFU. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.

Data are a combination of at least two separate experiments.

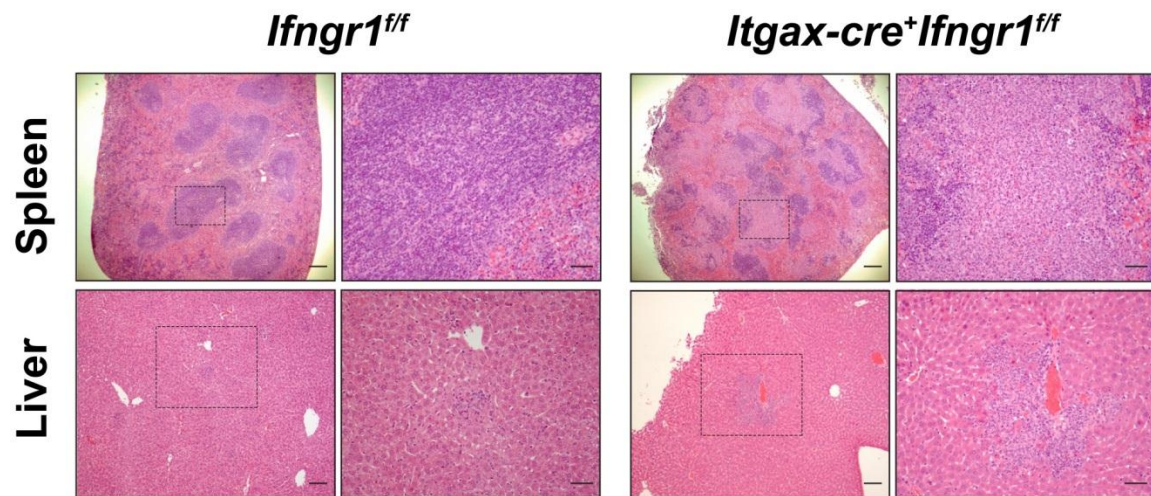


Figure 18. *Itgax-cre⁺Ifngr1^{f/f}* mice have increased histological lesions in spleen and liver

H&E staining of infected spleens and livers from mice at 5 days after infection (10^5 *Listeria* i.p.). Right panels show magnified regions (dashed boxes in left panels). Scale bar for spleen represents 200 μm in low-power images and 50 μm in high-power images; scale bar for liver represents 100 μm in low-power images and 50 μm in high-power images.

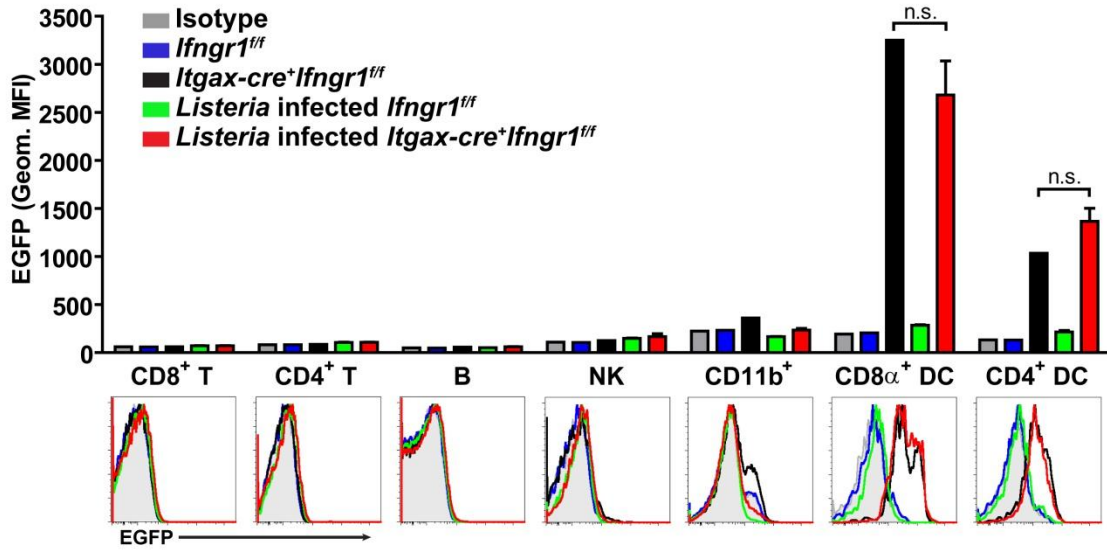


Figure 19. Bacterial infection itself induces no change in CD11c expression in *Itgax-cre⁺Ifngr1^{ff}* mice

Mice were i.p. infected with 10^5 *Listeria*. Spleens were harvested at 3 days after infection, gated as in Figure 1, and analyzed for expression of EGFP from *Ifngr1^{ff}* and *Itgax-cre⁺Ifngr1^{ff}*. Expression of EGFP is expected to have equimolar expression with cre recombinase because *Itgax* promoter directs bicistronic cre and EGFP protein expression. A graph shows geometric mean \pm SEM.

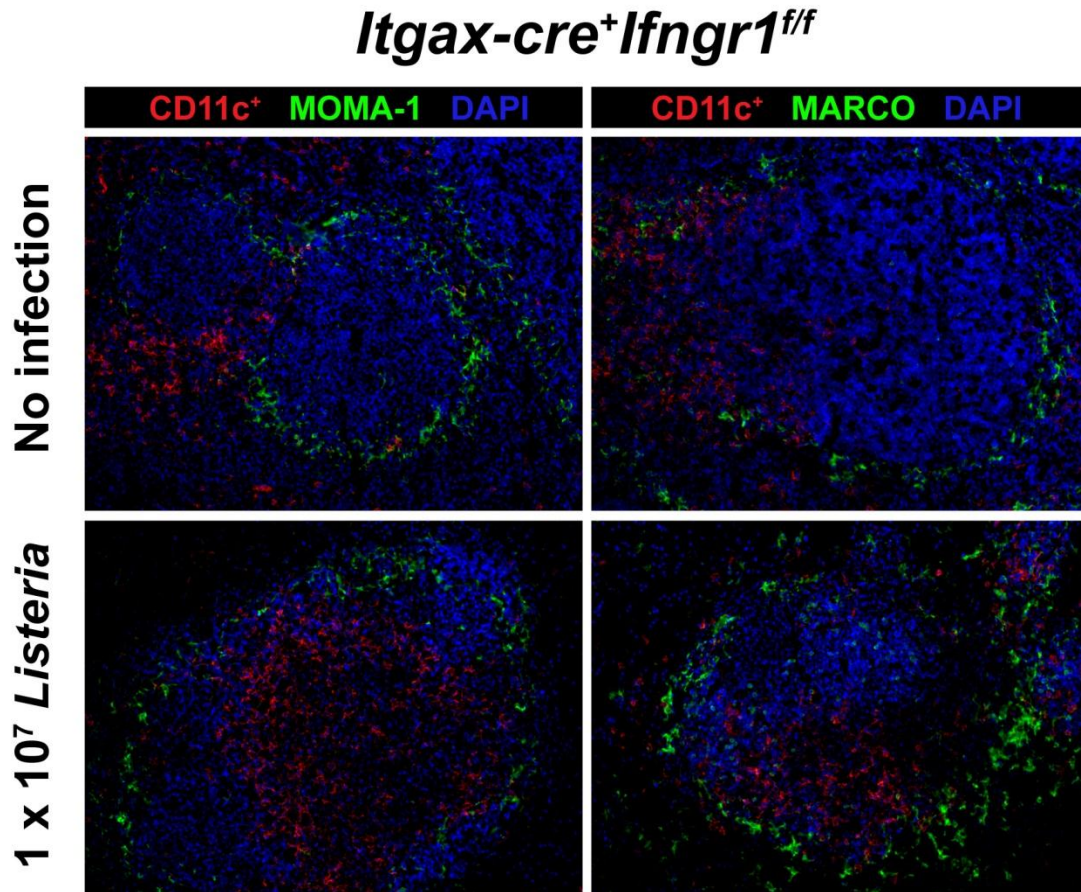


Figure 20. Marginal zone macrophages (MZM) and metallophilic MZM do not show up-regulation of CD11c expression by *Listeria* infection

Both *Ifngr1^{f/f}* and *Itgax-cre⁺Ifngr1^{f/f}* mice were i.v. infected with 10⁷ *Listeria*. After 18 hours of infection, spleen sections were harvested and stained with either MOMA-1 or MARCO (green), CD11c (red), and DAPI (blue).

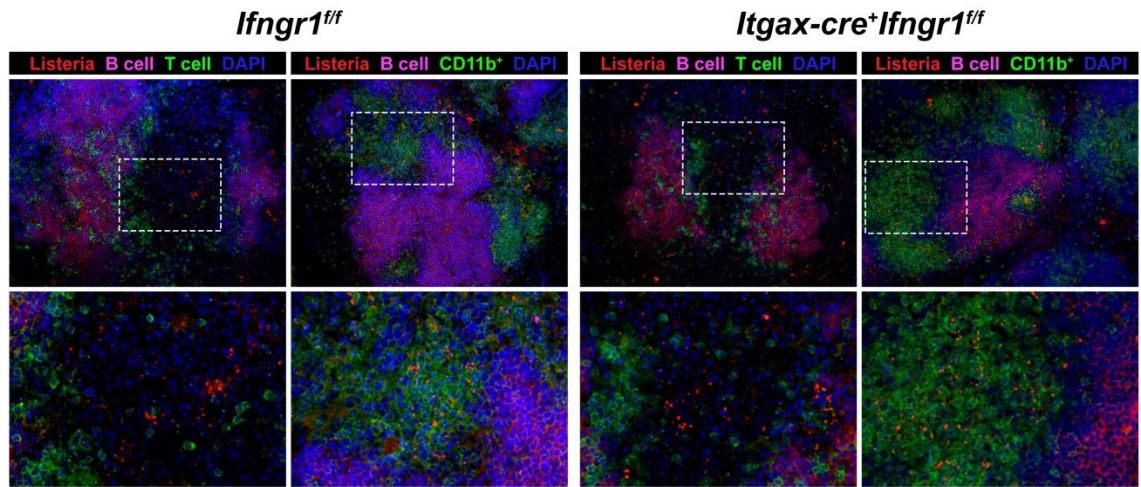


Figure 21. Immunostaining of splenic *Listeria* infection

Both *Ifngr1^{f/f}* and *Itgax-cre⁺Ifngr1^{f/f}* mice were i.v. infected with 10^7 *Listeria*. After 18 hours of infection, spleen sections were harvested and stained with *Listeria* O (red), B220 (pink), CD3 ϵ /CD11b (green), and DAPI (Blue). Lower panels show magnified regions (dashed boxes in upper panels).

CHAPTER 6

IFN γ from TNF α activated NK/NKT cells is required to prime CD8 α^+ DCs for optimal IL-12 production

IFN γ responsiveness in CD8 α^+ /CD103 $^+$ dendritic cells is required for early cytokine production

The serum amounts of cytokines and chemokine in *IfngrI^{ff}* and *Itgax-cre⁺IfngrI^{ff}* mice were measured to determine whether the crucial cytokines and chemokine were being produced normally in *Itgax-cre⁺IfngrI^{ff}* mice (Figure 22). In *IfngrI^{ff}* mice, serum IFN γ , IL-6, MCP-1, and TNF α production began to increase after 18 hours of infection, reached a peak at 18-72 hours, and decreased after 3 days of infection which nicely correlated with the changes of bacterial burden. In contrast, in *Itgax-cre⁺IfngrI^{ff}* mice these inflammatory cytokines and chemokine increased after day 3 of infection and remained at high amounts until day 10 of infection, which may reflect the increased bacterial burden in the *Itgax-cre⁺IfngrI^{ff}* mice compared to *IfngrI^{ff}* mice. Surprisingly, *Itgax-cre⁺IfngrI^{ff}* mice showed significantly decreased production of cytokines and chemokine at the early phase of infection, especially 18 hours, demonstrating that early systemic production of inflammatory cytokines and chemokine in *Itgax-cre⁺IfngrI^{ff}* mice is substantially delayed relative to *IfngrI^{ff}* mice. Therefore, I focused on identifying the defects in CD8 α^+ /CD103 $^+$ DCs that resulted in a decrease in cytokine responses during the early stages of *Listeria* infection.

IFN γ -insensitive CD8 α^+ DCs display defective IL-12 production

IL-12 is essential for resistance to sublethal doses of *Listeria* and for optimal IFN γ production by NK cells (Brombacher et al., 1999; Tripp et al., 1994). Previously, IL-12p35 $^{-/-}$ mice showed increases in the number and size of granulomatous lesions even at low doses of *Listeria* (≤ 1000 organisms). Moreover, CD8 α^+ DCs and monocytes have been implicated as major cellular sources of IL-12 in *Listeria* infection (Kapadia et al., 2011; Mitchell et al., 2011; Zhan et al., 2010). I therefore decided to examine IL-12 production by individual DC subsets during the first 24 hours of infection and determine what effects endogenously produced IFN γ has on IL-12 production by these cells. To address these issues, *ex vivo* intracellular cytokine staining was used to quantify the frequency of IL-12 positive cells in spleen at 6, 9, 12, 18, and 24 hours after *Listeria* infection (Figure 23). To obtain synchronized bacterial infections and generate enough cytokine-producing cells for *ex vivo* analysis, *IfngrI^{ff}* and *Itgax-cre⁺IfngrI^{ff}* mice were i.v. infected with a high dose of 10^6 *Listeria*. it was confirmed that i.v. infection of *Listeria* resulted in increased bacterial burdens in *Itgax-cre⁺IfngrI^{ff}* mice, similar to results obtained with i.p. infection, which indicates that the defects in *Listeria* clearance in *Itgax-cre⁺IfngrI^{ff}* did not depend on a specific infection route (Figure 24).

The percentage of splenic CD8 α^+ DCs producing IL-12p40 in *IfngrI^{ff}* mice was increased to 4.2% at 9 hours, reaching a peak level of 9.4% at 12 hours and 5.9% at 18 hours (Figure 23A and B). In contrast, the proportion of IL-12p40 producing CD8 α^+ DCs from *Itgax-cre⁺IfngrI^{ff}* mice was significantly less (64% reduction at 9 hours, 74% reduction at 12 hours, and 89% reduction at 18 hours). IL-12 production by CD8 α^+ DCs

in *Ifngr1^{wu}*^{-/-} mice was also examined at 9 hours post-infection (Figure 25). Surprisingly, IL-12p40 producing CD8 α ⁺ DCs from *Ifngr1^{wu}*^{-/-} mice was completely absent. I interpret finding as indicating that CD8 α ⁺ DCs absolutely require IFN γ responsiveness for IL-12p40 production, and incomplete reduction in IL-12p40⁺CD8 α ⁺ DC is due to incomplete deletion of *Ifngr1* in *Itgax-cre*⁺*Ifngr1^{ff}* mice

IL-12p40 production by CD8 α ⁺ DCs subsided 24 hours after infection. However, at this time, approximately 10% of Ly6C^{hi} monocytes stained positively for IL-12p40 in both *Ifngr1^{ff}* and *Itgax-cre*⁺*Ifngr1^{ff}* mice (Figure 23A and C). Recently, Ly6C^{hi} inflammatory monocytes were shown to be recruited from the bone marrow to site of *Listeria* infection and differentiated into TNF α and iNOS producing dendritic cells (TipDC) (Serbina and Pamer, 2006; Serbina et al., 2003). Thus, I further established the relationship of IL-12p40⁺Ly6C^{hi} monocytes and TNF α ⁺iNOS⁺Ly6C^{hi} TipDCs (Figure 26). Approximately 30% of IL-12p40⁺Ly6C^{hi} monocytes had a phenotype ascribed to TipDCs—identified by their production of TNF α and iNOS and 60% of TipDCs produced IL-12p40 at this time. Therefore, CD11c^{int}CD11b⁺Ly6C^{hi}Ly6G⁻ monocytes displayed a temporally delayed production of IL-12p40 compared to CD8 α ⁺ DCs.

IFN γ insensitivity in CD8 α ⁺ DCs leads to a reduction of IFN γ from NK/NKTs

NK and NKT cells were identified as the major sources of IFN γ production during the first 24 hours of *Listeria* infection (Figure 27). IL-12 is known to be required for early IFN γ production by NK cells (Tripp et al., 1993). Therefore the consequences of IFN γ unresponsiveness in CD8 α ⁺ DCs on the early induction of IFN γ was examined.

The percentage of IFN γ ⁺ NK/NKT cells in *Itgax-cre*⁺*Ifngr*^{ff} mice was significantly decreased compared to that in *Ifngr*^{ff} mice at both 9 and 12 hours after infection (NK cells: 68% reduction at 9 hours and 25% reduction at 12 hours; NKT cells: 58% reduction at 9 hours and 35% reduction at 12 hours) (Figures 27A-C). The reduced percentage of IFN γ ⁺ NK/NKT cells from *Itgax-cre*⁺*Ifngr*^{ff} mice occurred concomitantly with the decreased percentage of IL-12p40⁺CD8 α ⁺ DCs (Figure 23). However, the levels of IFN γ ⁺ NK/NKT cells in *Itgax-cre*⁺*Ifngr*^{ff} mice recovered to the those found in *Ifngr*^{ff} mice by 18 to 24 hours when Ly6C^{hi} inflammatory monocytes produced the second wave of IL-12p40. CD4⁺ DCs, plasmacytoid DCs, CD4⁺ T cells, B cells, macrophages, and neutrophils were not a significant source of either IL-12 or IFN γ in the first 24 hours of *Listeria* infection (Figure 28).

NK/NKT cells provide IFN γ to CD8 α ⁺ DCs for optimal IL-12 production

Our data show direct effects of IFN γ on CD8 α ⁺ DCs, and therefore I reasoned that there may be an interacting cellular loop between NK cells and CD8 α ⁺ DCs. Specifically it was explored whether NK/NKT cells first produced IFN γ that primes CD8 α ⁺ DCs for the first wave of IL-12 production. To test this idea, NK/NKT cells was depleted by treating *Ifngr*^{ff} mice with NK1.1 mAb, and asked whether there was a corresponding decrease in IL-12 production by CD8 α ⁺ DCs (Figure 29A). To prevent the possibility that increased bacterial burdens caused by NK/NKT depletion might directly affect cytokine production, IL-12p40 at 9 hours after infection was measured. This was the earliest time point at which IL-12p40 production by CD8 α ⁺ DCs could be detected

(Figure 23). It also preceded the exponential growth phase of *Listeria* (Carrero et al., 2009). In mice treated with anti-NK1.1 mAb both NK and NKT cells were depleted, and the percentage of IL-12p40⁺CD8 α ⁺ DCs was 60% decreased (Figure 29B). These results point to a crosstalk between NK/NKT cells and DCs, as IFN γ from NK/NKT cells is responsible for initiating IL-12 production from CD8 α ⁺ DCs, and DC-produced IL-12 then stimulates more IFN γ production from NK/NKT cells.

CD8⁺ T cells were also identified as a subsequent source of IFN γ production at 18 hours although the percentages of IFN γ ⁺ CD8⁺ T cells were much smaller than those of NK/NKT cells (Figure 27A and D). As memory phenotype (MP) CD8⁺ T cells were shown to produce IFN γ without their cognate antigens after *Listeria* infection (Berg et al., 2003), IFN γ ⁺ CD8⁺ T cells resembled previously reported MP CD8⁺ T cells, as they were CD44^{hi}CXCR3⁺CD62L^{hi} (Figure 30). A recent study demonstrated that MP CD8⁺ T cells secreted IFN γ two hours after *S. Typhimurium* infection, however I failed to detect IFN γ production in MP CD8⁺ T cells until 18 hours after *Listeria* infection (Kupz et al., 2012). It was possible that very early IFN γ production by MP CD8⁺ T cells occurred and was below the detection limit of flow cytometry but might provide another source of IFN γ that activated CD8 α ⁺ DCs. To address this idea, MP CD8⁺ T cells were depleted using a CXCR3 mAb developed in our lab (Uppaluri et al., 2008). Using our hamster CXCR3-173 mAb, we previously showed that CXCR3 was expressed most highly in MP CD8⁺CD44⁺ T cells, and *in vivo* treatment with anti-CXCR3 mAb selectively depleted MP CD8⁺CD44⁺CD122⁺ T cells (Figure 31). Mice treated with CXCR3-173 mAb exhibited no reduction in IL-12p40 production (Figure 29B) despite complete elimination

of the MP CD8⁺ T cell population. Further, combined treatment with CXCR3-173 mAb and anti-NK1.1 mAb showed no further inhibition of IL-12 production from that seen with anti-NK1.1 mAb alone. These results indicate that memory phenotype CD8⁺ T cells are not the source of the early IFN γ that primes CD8 α^+ DCs for IL-12p40 production.

TNF α initiates the reciprocal activation of NK/NKTs and CD8 α^+ DCs to produce IFN γ and IL-12

While the results presented thus far show that optimal early IL-12 production from CD8 α^+ DCs is a consequence of early IFN γ production by NK/NKT cells, they did not identify the cytokine(s) responsible for the IFN γ that initiates the entire process. As TNF α , IL-18, and IL-1 are known to induce IFN γ from NK cells upon *Listeria* infection (Humann and Lenz, 2010; Tripp et al., 1993; Tsuji et al., 2004), I analyzed IFN γ and IL-12p40 production after treating *Ifngr*^{ff} mice with neutralizing antibodies specific either for these cytokines or their receptors and infecting with *Listeria* (Figure 32). It was found that the reciprocal activation of NK/NKT cells and CD8 α^+ DCs after *Listeria* infection was TNF α -dependent. To insure that both soluble and cell surface TNF α were neutralized, mAbs specific for each of the two TNF receptors (TNFR) were used. Dual blockade of TNFR1 and TNFR2 caused a 54% and 79% decrease in IFN γ^+ NK and NKT cells, respectively, which was accompanied by a 75% reduction in IL-12p40⁺CD8 α^+ DCs. Direct neutralization of IFN γ using a IFN γ specific mAb achieved a comparable 78% decrease in IL-12p40⁺CD8 α^+ DCs (Figure 32A). In contrast, IL-18 neutralization had no effect on IL-12p40 production, although IL-18 neutralizing mAb in combination with

TNFR1 plus TNFR2 blocking mAbs down-regulated IFN γ ⁺ NK cells 23% more than dual TNFR-blockade alone (Figure 32B). A cocktail of neutralizing IL-1 α , IL-1 β and blocking IL-1R1 mAbs caused a 30% decrease in IL-12p40⁺CD8 α ⁺ DCs without altering the percentage of IFN γ ⁺ NK/NKT cells (Figure 32A). Thus, the initial production of IFN γ from NK/NKT cells early in *Listeria* infection that sets in motion a reciprocal activation of NK/NKT cells and CD8 α ⁺ DCs is a consequence of a process induced predominantly by TNF α with potential participation of other early arising pro-inflammatory cytokines.

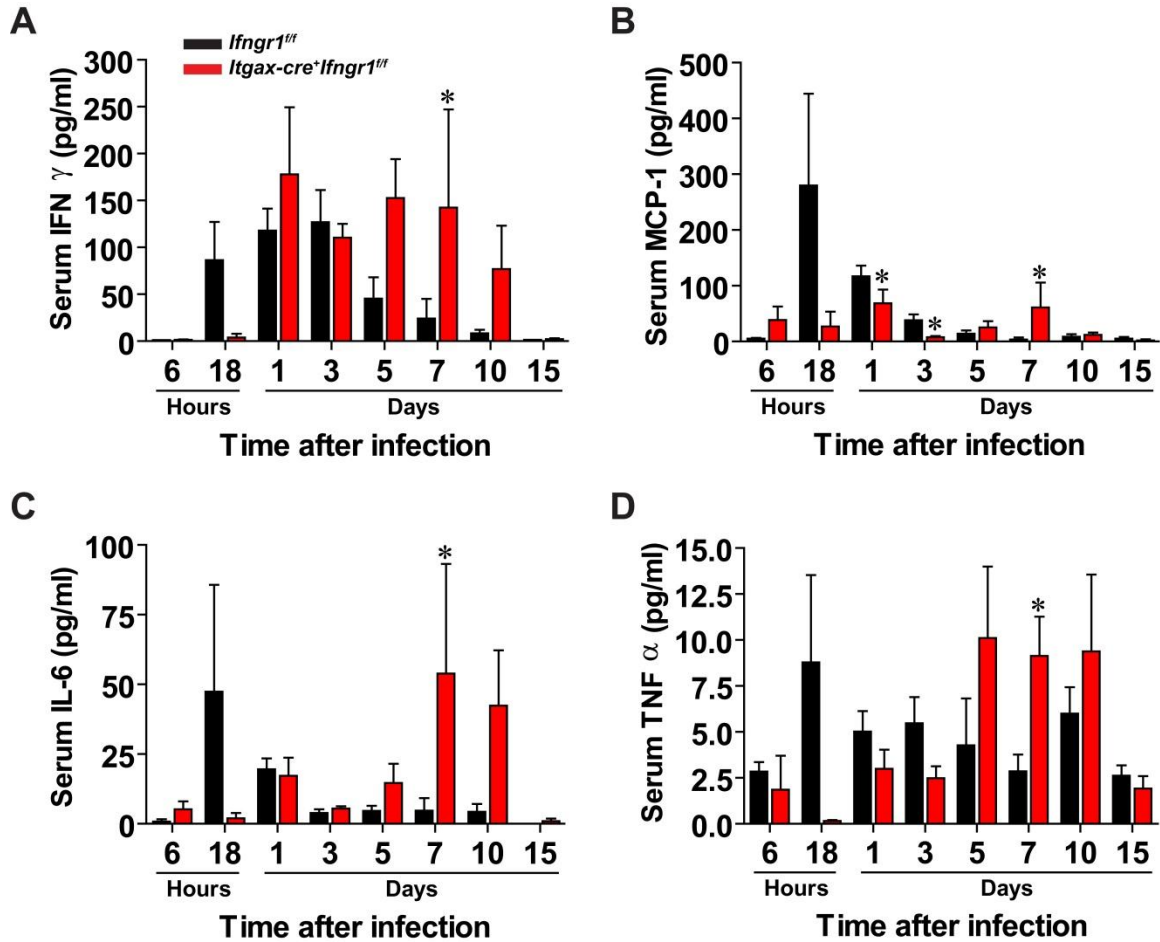


Figure 22. $Itgax-cre^{+}Ifngr1^{ff}$ mice show delayed production of early pro-inflammatory cytokines and chemokine

Infected $Ifngr1^{ff}$ and $Itgax-cre^{+}Ifngr1^{ff}$ were bled at indicated time points after infection, and serum was analyzed for IFN γ (A), MCP-1 (B), IL-6 (C), and TNF α (D) concentration using CBA. Data represent averages of serum concentration of cytokines/chemokine through the course of infection from at least two separated experiments ($n \geq 4$ at each time point).

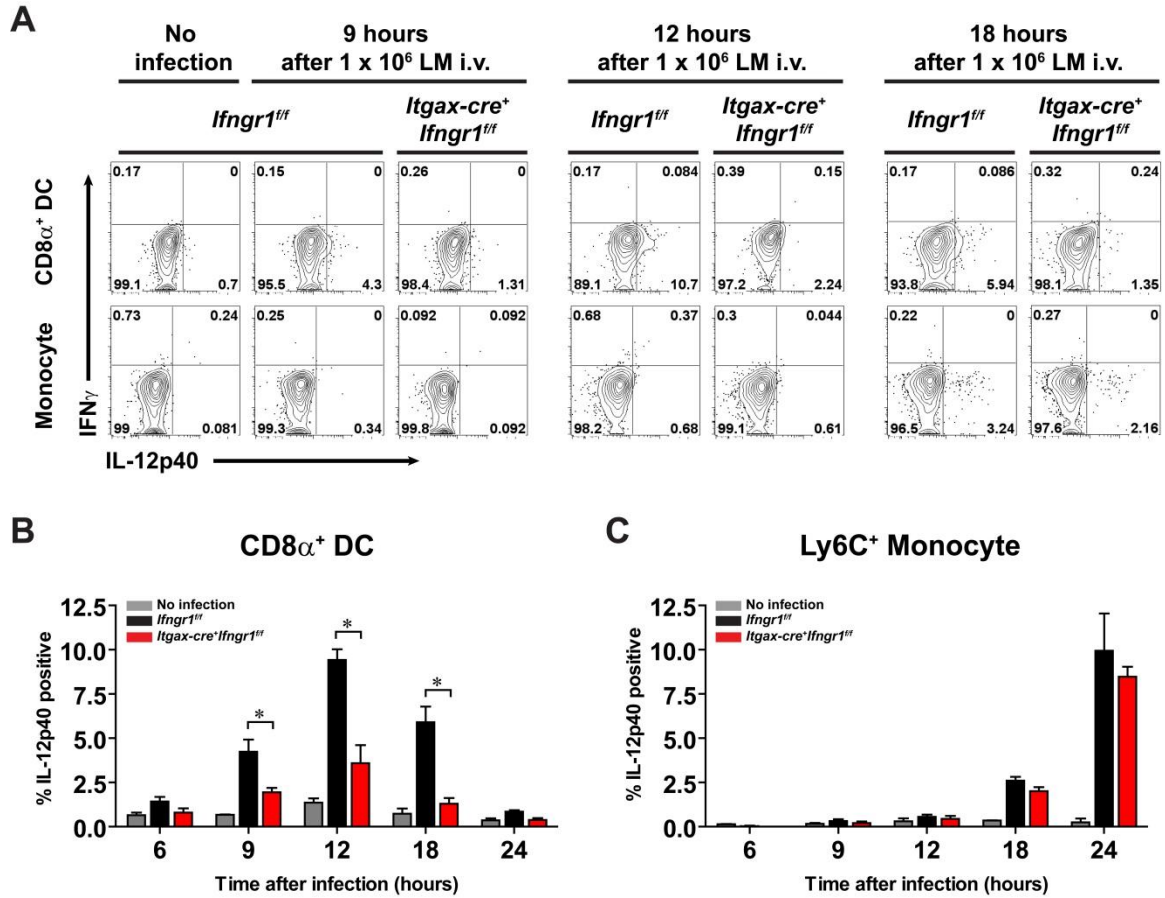


Figure 23. The early production of IL-12p40 from CD8 α ⁺ DCs is significantly decreased in *Itgax-cre⁺Ifngr1^{ff}* mice

Itgax-cre⁺Ifngr1^{ff} and *Ifngr1^{ff}* mice were i.v. infected with 10⁶ *Listeria*. Spleens were harvested at 6, 9, 12, 18, and 24 hours after infection, and analyzed for the cellular source of IL-12p40 by intracellular cytokine staining.

(A) Representative flow cytometry plots for IL-12p40 expression from cells gated on CD11c⁺Dec205⁺CD8 α ⁺ DCs and CD11b⁺Ly6C⁺Ly6G⁻ monocytes. For each quadrant, gating was based on cells from uninfected controls that were performed at every time point (For simplicity, only the uninfected control at 9 hours after infection is shown).

(B and C) Percentages of IL-12p40 positive CD8 α^+ DCs, IL-12p40 positive Ly6C $^+$ monocytes in the spleen during 24 hours of *Listeria* infection ($n \geq 4$ at each time point). *, $p \leq 0.05$.

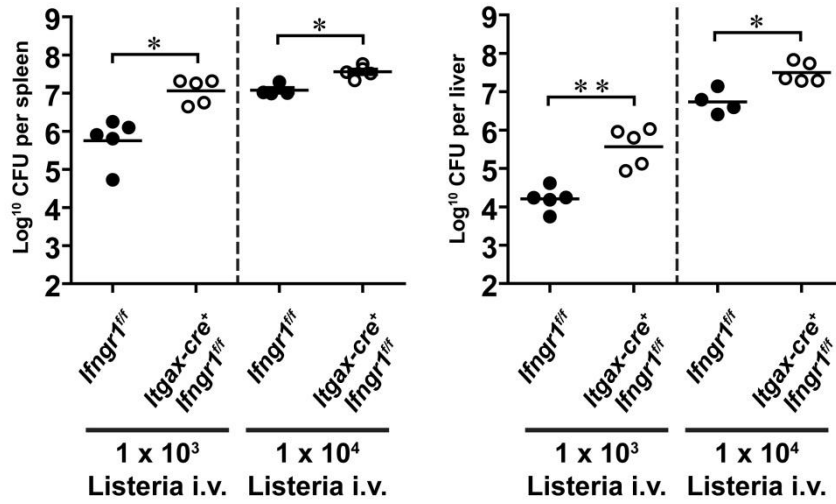


Figure 24. *Itgax-cre⁺Ifngr1^{f/f}* mice show increased bacterial burden after intravenous infection

Listeria CFUs per spleen and liver of *Ifngr1^{f/f}* and *Itgax-cre⁺Ifngr1^{f/f}* mice at 3 days after i.v. infection with 10³ or 10⁴ *Listeria*. Each symbol represents an individual mouse. Lines represent the mean Log₁₀CFU. *, p ≤ 0.05; **, p ≤ 0.01.

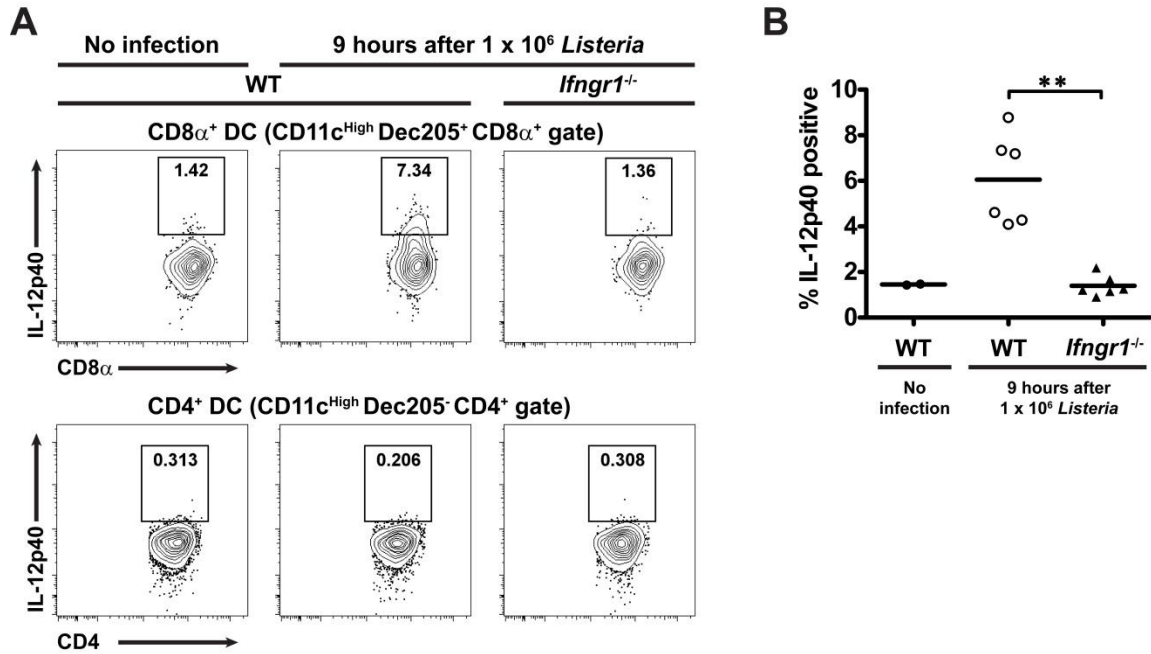


Figure 25. The early production of IL-12p40 from CD8 α ⁺ DCs is completely abrogated in *Ifngr1*^{WU}^{-/-} mice

Itgax-cre⁺ *Ifngr1*^{f/f} and *Ifngr1*^{f/f} mice were i.v. infected with 10^6 *Listeria*. Spleens were harvested at 9 hours after infection and analyzed for the cellular source of IL-12p40 by intracellular cytokine staining.

(A) Representative flow cytometry plots for IL-12p40 expression from cells gated on CD11c⁺Dec205⁺CD8 α ⁺ DCs and CD11c⁺Dec205⁻CD4⁺ DCs.

(B) Percentages of IL-12p40 positive CD8 α ⁺ DCs in the spleen after 9 hours infection. **, $p \leq 0.01$.

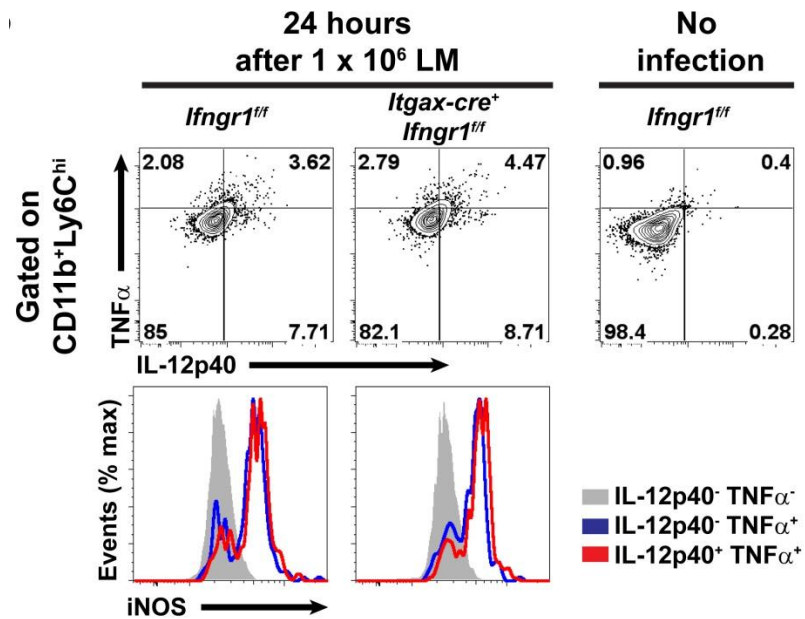


Figure 26. *Listeria* infection induces TipDCs to produce IL-12p40

At 24 hours after i.v. infection with 10^6 *Listeria*, *Ifngr1^{fl/fl}* and *Itgax-cre⁺Ifngr1^{fl/fl}* mice were analyzed for TNF α and iNOS expression on IL-12p40 producing monocytes.

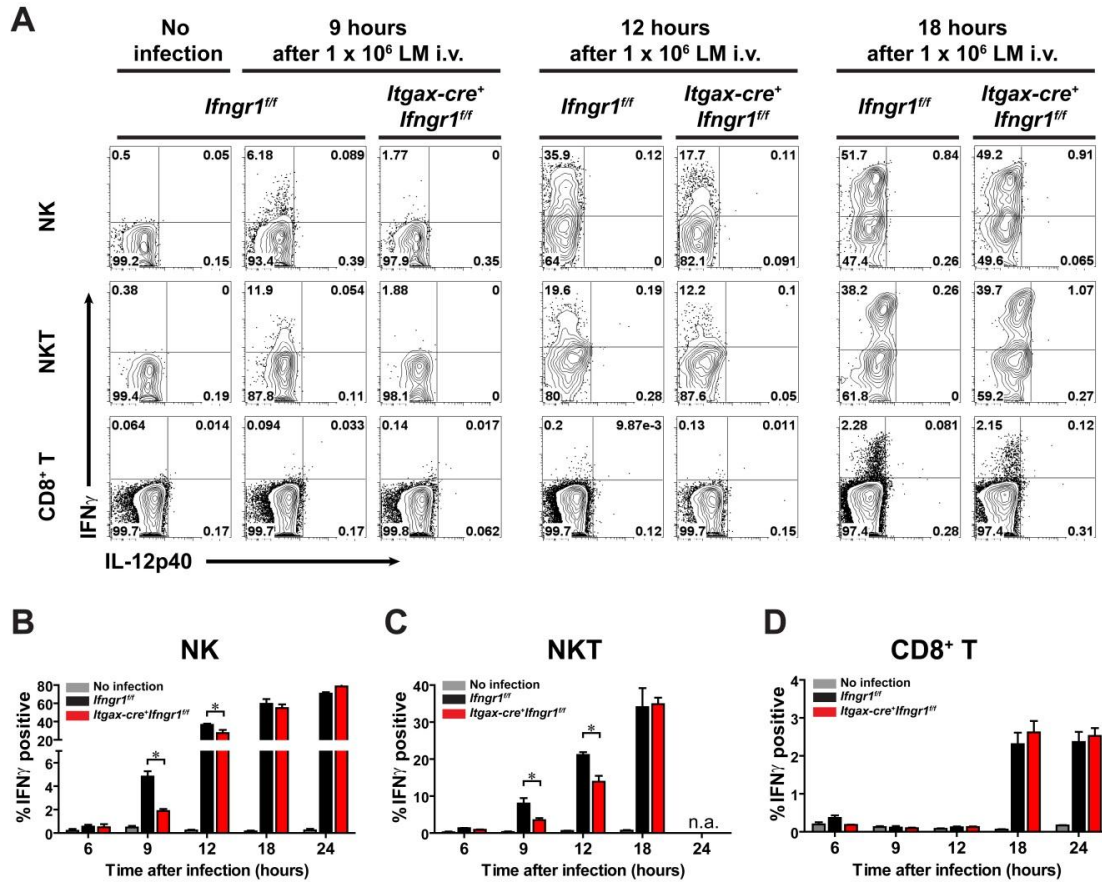


Figure 27. The early production of IFN γ from NK and NKT cells is significantly decreased in *Itgax-cre⁺Ifngr1^{fl/fl}* mice

Itgax-cre⁺Ifngr1^{fl/fl} were i.v. infected with 10⁶ *Listeria*. The cellular sources of IFN γ in spleen were analyzed by intracellular cytokine staining at 6, 9, 12, 18, and 24 hours after infection.

(A) Representative plots for IFN γ which were gated on CD3 ϵ ⁺NK1.1⁺ NKs, CD3 ϵ ^{int}NK^{int} NKTs, and CD3 ϵ ⁺CD8⁺ T cells.

(B) Percentages of IFN γ positive NKs, NKTs, CD8⁺ T cells in the spleen during 24 hours of infection (n \geq 4 at each time point). *, p \leq 0.05.

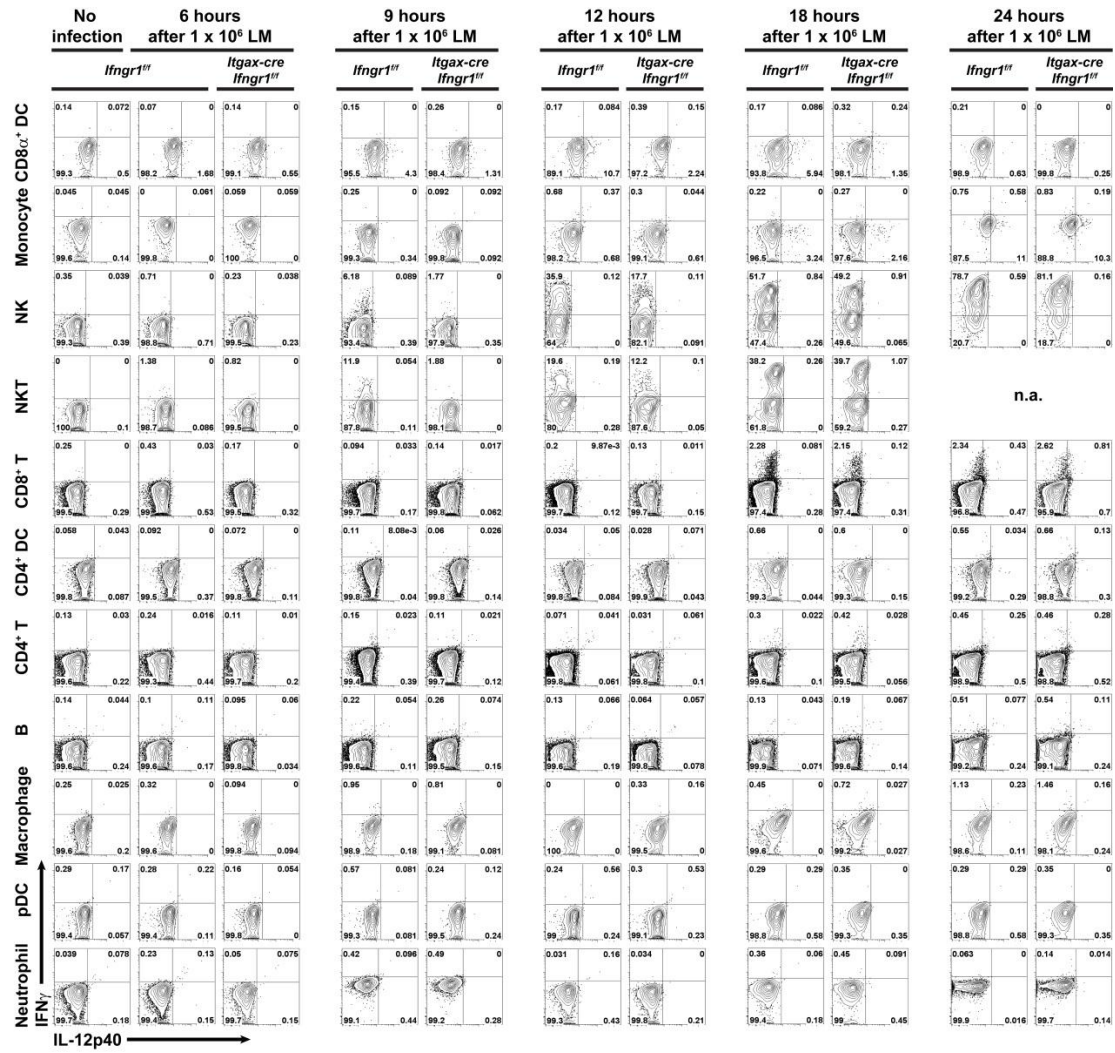


Figure 28. Flow cytometry analysis for IL-12 and IFN γ through the course of infection

Ifngr1^{ff} and *Itgax-cre⁺Ifngr1^{ff}* were i.v. infected with 10⁶ *Listeria*. Spleens were harvested at 6, 9, 12, 18, and 24 hours after infection, gated as in Figure 1, and analyzed for the cellular source of IL-12 and IFN γ by intracellular cytokine staining. Data are representative FACS plots. Each quadrant gating was based on no infection controls that were done at every time point (only no infection control at 6 hours after infection is shown for simplicity).

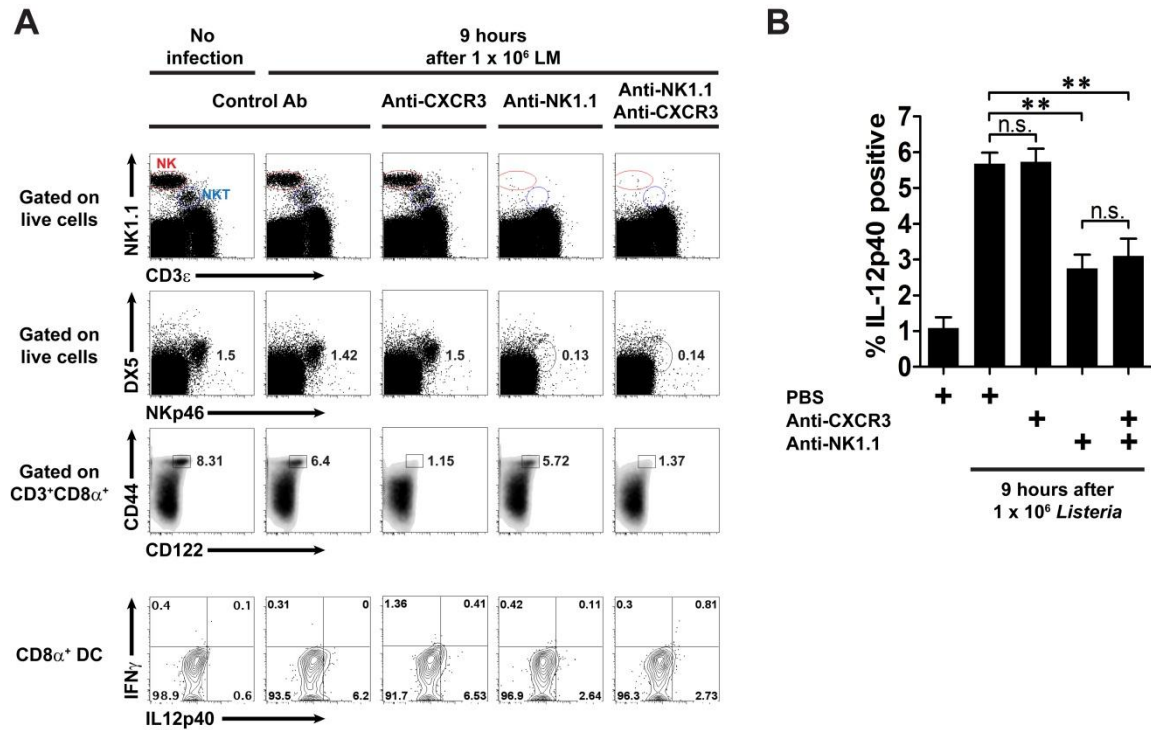


Figure 29. Depletion of NK/NKT cells significantly reduces IL-12p40 production from $CD8\alpha^+$ DCs

Ifngrl^{ff} and *Itgax-cre⁺Ifngrl^{ff}* mice treated with indicated mAbs were i.v. infected with 10^6 *Listeria*. Spleens were harvested at 9 hours after infection, and analyzed for IL-12p40 production from $CD8\alpha^+$ DCs by intracellular cytokine staining.

(A) Representative FACS plots for the depletion of NK/NKT cells by anti-NK1.1 mAb, for the depletion of MP $CD8^+$ T cells by anti-CXCR3 mAb, and for IL-12p40 production from $CD8\alpha^+$ DCs after depleting mAb treatments.

(B) Summary of percentages of IL-12p40 positive $CD8\alpha^+$ DCs after depleting mAb treatments. **, $p \leq 0.01$.

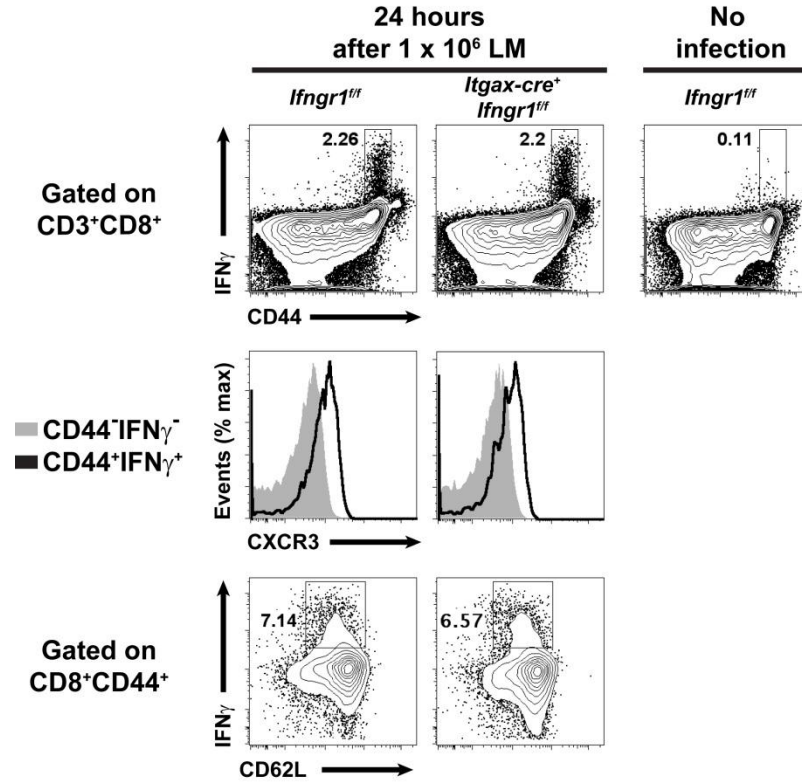


Figure 30. *Listeria* infection induces memory phenotype CD8⁺ T cells to produce IFN γ

At 24 hours after i.v. infection with 10^6 *Listeria*, *Ifngr1^{ff}* and *Itgax-cre⁺Ifngr1^{ff}* mice were analyzed for CD44, CXCR3, and CD62L on IFN γ producing CD8⁺ T cells by flow cytometry.

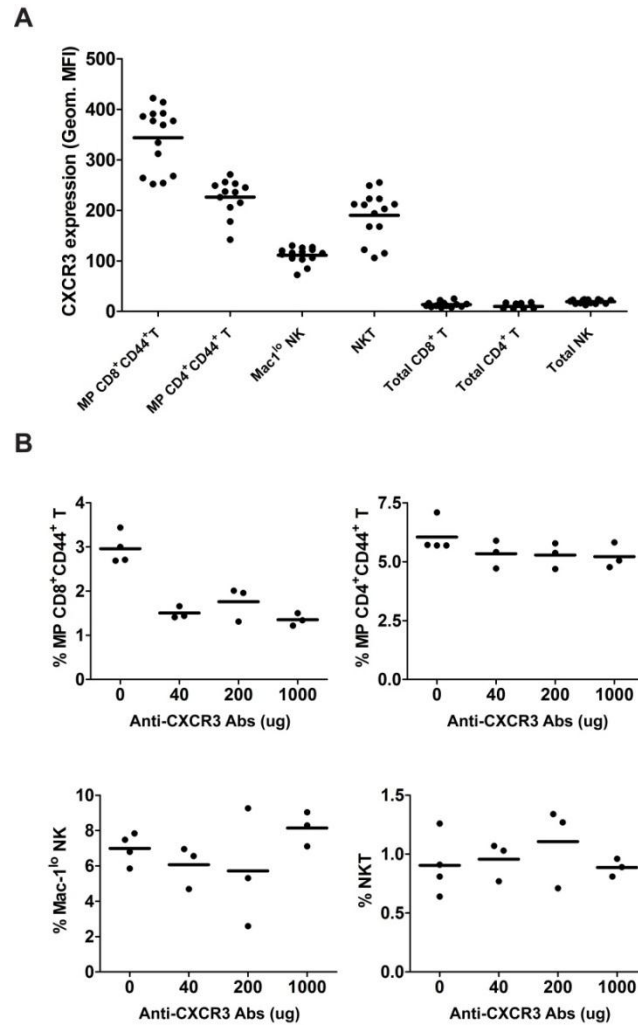


Figure 31. Selective depletion of memory phenotype CD8⁺ T cells by anti-CXCR3 mAb

(A) The summarized CXCR3 expression in the indicated cellular subsets to show the strongest expression of CXCR3 in memory phenotype (MP) CD8⁺ T cells of B6 WT mice.

(B) B6 WT mice were i.p. injected with 40, 200, and 1000 µg of anti-CXCR3, and 3 days later the frequency of MP CD8⁺ T, MP CD4⁺ T, NK, and NKT cells were analyzed by flow cytometry.

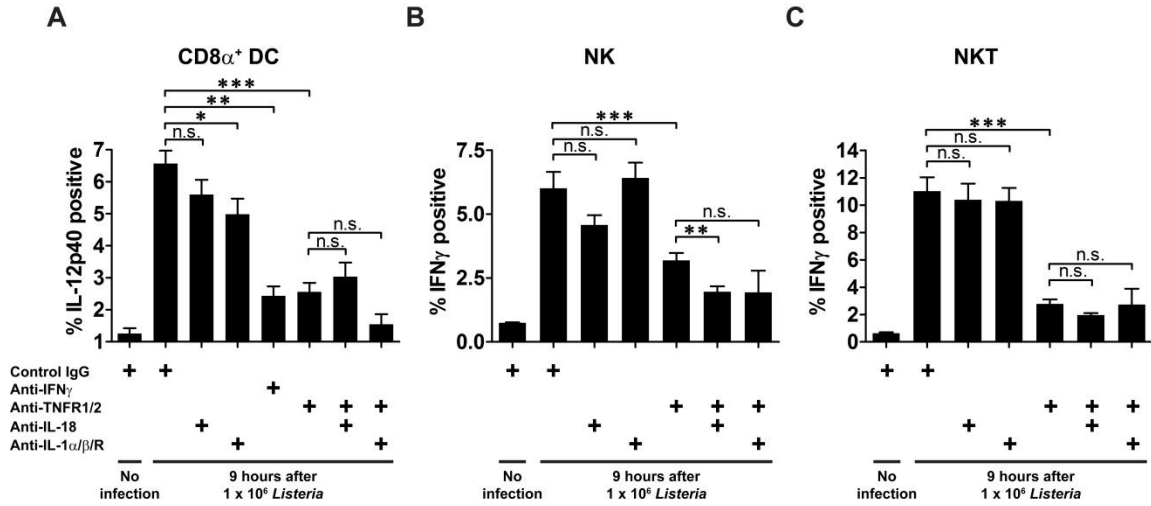


Figure 32. Neutralization of TNF α reduces both IL-12 production from CD8 α^+ DCs and IFN γ production from NK/NKT cells

Ifngrl^{ff} and *Itgax-cre⁺Ifngrl^{ff}* mice treated with indicated mAbs were i.v. infected with 10⁶ *Listeria*. Spleens were harvested at 9 hours after infection, and analyzed for the cellular source of IL-12p40 and IFN γ by intracellular cytokine staining. Percentages of IL-12p40 positive CD8 α^+ DCs (A), IFN γ positive NKs (B), and IFN γ positive NKTs (C) in the spleen were plotted as bar graphs. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

CHAPTER 7

IFN γ responsiveness in neutrophils is required to control *Listeria* infection

***LysM-cre⁺Ifngr1^{ff}* mice show a relatively selective IFN γ unresponsiveness in neutrophils**

Next, *Ifngr1^{ff}* mice were bred with *LysM-cre* mice to obtain macrophage/neutrophil specific *Ifngr1* deletion. As expected, both resident peritoneal macrophages and neutrophils are sensitive to cre-mediated deletion under the control of LysozymeM promoter (Figure 33A). However, it is noteworthy that IFNGR1 expression on red pulp macrophages in the spleen and kupffer cells in liver was not affected. Having substantially diminished IFNGR1 expression in resident peritoneal macrophages and neutrophils, the level of pSTAT1 in these cells was measured as functional readouts after *in vitro* IFN γ treatment. In *LysM-cre⁺Ifngr1^{ff}* mice, both resident peritoneal macrophages and neutrophils showed significant inhibition of STAT1 phosphorylation by IFN γ (Figure 33B).

***LysM-cre⁺Ifngr1^{ff}* mice succumb to *Listeria* infection**

LysM-cre⁺Ifngr1^{ff} mice were infected to test their susceptibility to *Listeria*. After challenging with a sub-lethal dose of *Listeria* (1×10^5), half of the *LysM-cre⁺Ifngr1^{ff}* mice died within 2 weeks after infection while most of *Ifngr1^{ff}* mice survived (Figure 34A). When *LysM-cre⁺Ifngr1^{ff}* mice were challenged and bacterial titers in spleens and livers from the survivors were monitored, *LysM-cre⁺Ifngr1^{ff}* mice exhibited significantly

increased *Listeria* burdens compared to infected control mice (Figure 34B). Increased bacterial colonization was more prominent in the liver than the spleen in *LysM-cre⁺Ifngr1^{ff}* mice. Increased *Listeria* burdens in liver were observed at the early phase of infection, with a 6-fold increase in CFUs over *Ifngr1^{ff}* mice at day 1, and were heightened up to 17,000-fold increase at day 7. In contrast, there was no difference in early splenic bacterial titers at day1 and 3.

We previously demonstrated that IFNGR1 expression on peritoneal macrophages is essential for host survival during LM infection (Dighe et al., 1995). However, it remains unclear whether the increased susceptibility of *LysM-cre⁺Ifngr1^{ff}* mice is due to IFN γ insensitivity in neutrophils. Recently, it has been shown that neutrophils are specifically required for optimal clearance of *Listeria* from liver using the Ly6G mAb that selectively depletes Ly6G⁺ neutrophils (Carr et al., 2011). In these neutrophil-depleted mice, bacterial clearance in spleen was less dependent on neutrophils than in liver. This observation is consistent with my findings that *LysM-cre⁺Ifngr1^{ff}* mice had a more prominent bacterial infection in liver than in spleen. To explore whether neutrophils require IFN γ sensitivity in more detail, I adoptively transferred WT or *Ifngr1^{-/-}* neutrophils into *LysM-cre⁺Ifngr1^{ff}* mice and asked whether it could rescue the *Listeria* sensitivity of these mice (Figure 35A and B). Surprisingly, WT neutrophils completely restored the resistance of *LysM-cre⁺Ifngr1^{ff}* mice to *Listeria* infection in spleen and liver, whereas *Ifngr1^{-/-}* neutrophils did not (Figure 35C).

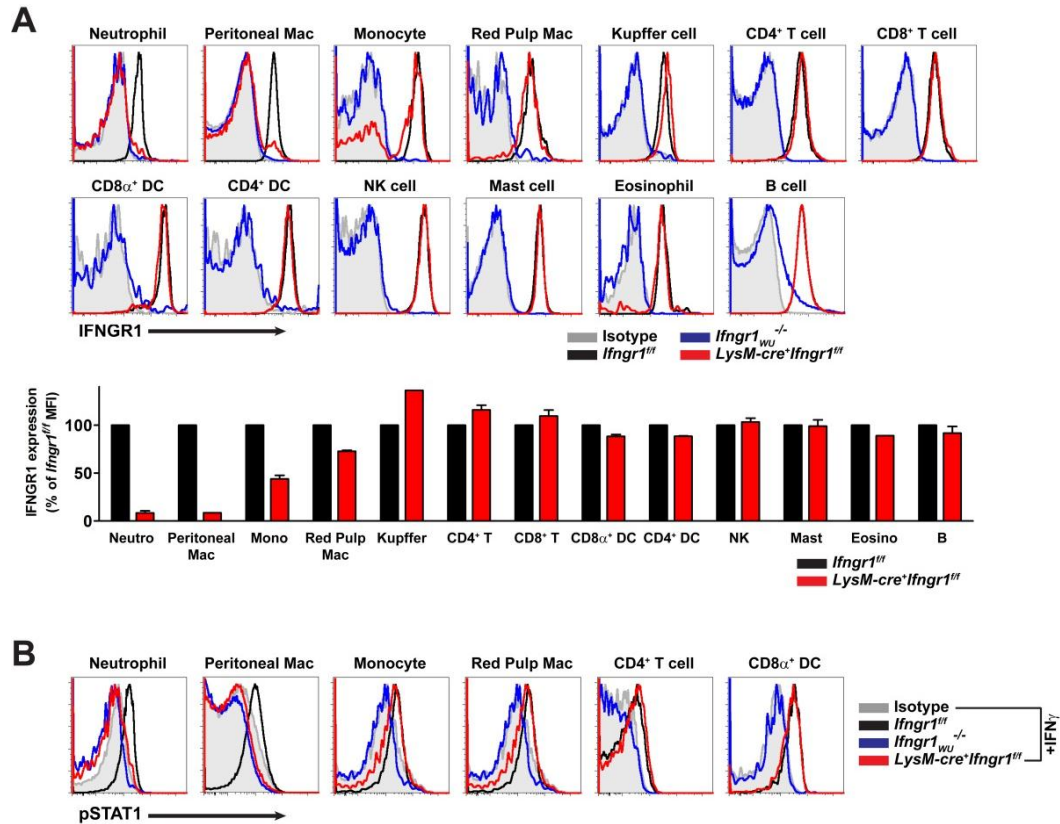


Figure 33. *LysM-cre⁺Ifngr1^{f/f}* has no functional IFNGR1 in neutrophils and peritoneal macrophages.

(A) IFNGR1 expression on various hematopoietic cells was measured using flow cytometry in *LysM-cre⁺Ifngr1^{f/f}* mice. The spleen/PBLs/liver/peritoneal cavity from *Ifngr1^{f/f}*, *LysM-cre⁺Ifngr1^{f/f}*, and *Ifngr1^{WU/-}* were harvested and stained with either biotinylated anti-IFNGR1 mAb or Isotype Ab with various lineage markers (Figure 1). IFNGR1 levels in the indicated cellular subsets in *LysM-cre⁺Ifngr1^{f/f}* mice compared with *Ifngr1^{f/f}* mice were summarized in bar graph. (B) Measurement of phosphorylated STAT1 in splenocytes from *LysM-cre⁺Ifngr1^{f/f}* mice after *in vitro* IFN γ treatment (1,000 U/ml) for 15 minutes at 37°C.

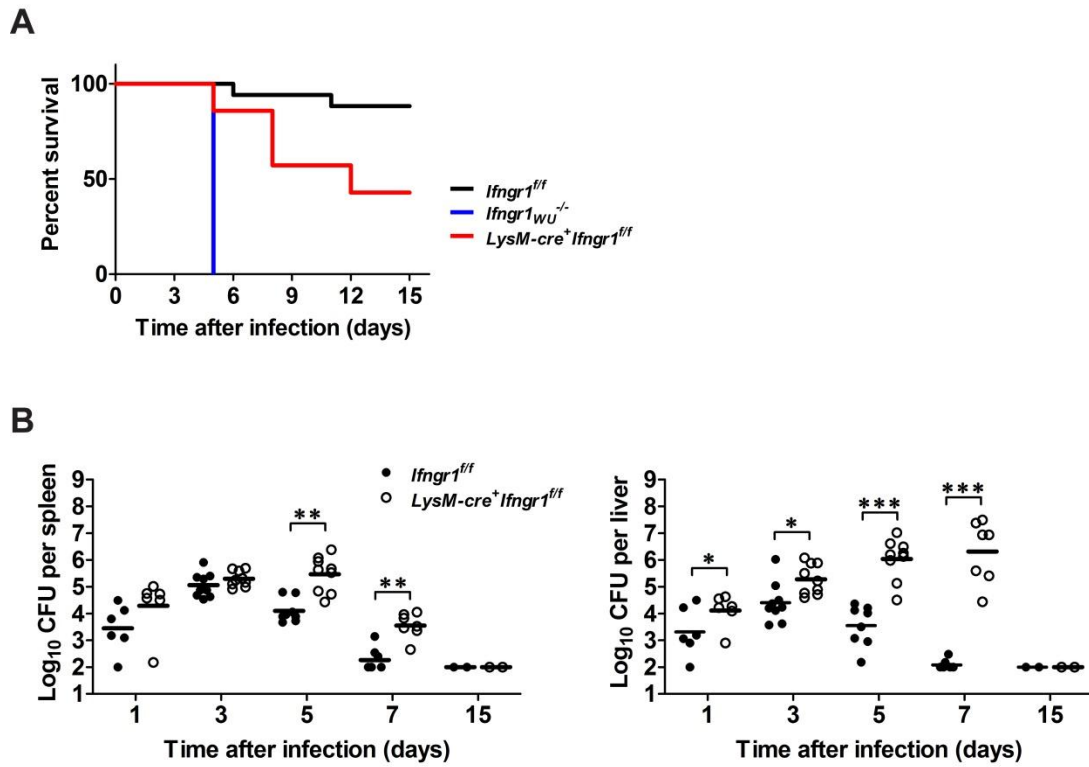


Figure 34. *LysM-cre⁺Ifngr1^{fl/fl}* mice are susceptible to *Listeria* infection

(A) Survival of mice which were i.p. infected with 10^5 *Listeria*. (B) *Listeria* CFUs per spleen and liver of *Ifngr1^{fl/fl}*, *LysM-cre⁺Ifngr1^{fl/fl}*, and *Ifngr1^{WU-/-}* mice which were i.p. infected with 10^5 *Listeria*. Each symbol represents an individual mouse. Lines represent the mean Log₁₀CFU. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

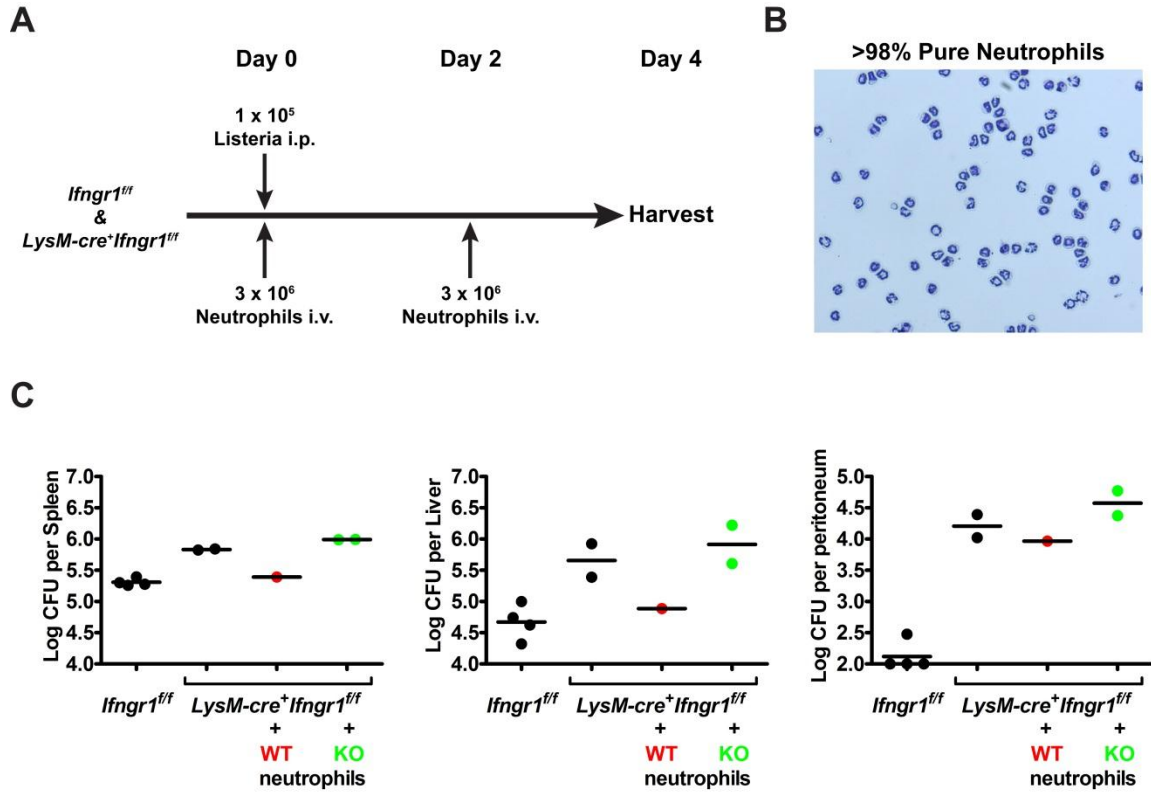


Figure 35. Adoptive transfer of WT neutrophils rescues *LysM-cre⁺Ifngr1^{ff}* mice from *Listeria* infection

(A and B) Neutrophils were isolated from WT and *Ifngr1^{-/-}* bone marrows and were adoptively transferred into *Listeria* infected *LysM-cre⁺Ifngr1^{ff}* mice at day 0 and 2 as shown. (C) *Listeria* CFUs per spleen, liver, and peritoneal cavity of *Ifngr1^{ff}*, *LysM-cre⁺Ifngr1^{ff}*, WT neutrophils-transferred *LysM-cre⁺Ifngr1^{ff}*, and KO neutrophils-transferred *LysM-cre⁺Ifngr1^{ff}* mice. Each symbol represents an individual mouse. Lines represent the mean Log₁₀CFU. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

CHAPTER 8

Discussion and future directions

The role of IFN γ in augmenting the production of IL-12 by myeloid cells was initially suggested by *in vitro* studies showing that pretreatment of macrophages with IFN γ before addition of heat-killed *Listeria* (HKLM) greatly enhanced their capacity to produce IL-12 (Dighe et al., 1995). This conclusion was also supported by other *in vitro* studies that used the macrophage-like RAW 264.7 cell line to show that IFN γ enhanced transcription of the genes encoding both IL-12p40 and IL-12p35 (Trinchieri, 2003). In contrast, the initial production of IL-12 by CD8 α^+ DC was found to be independent of IFN γ at least in response to *Toxoplasma gondii* (Scharton-Kersten et al., 1996). However, despite the significant amount of data pointing to a critical interaction between IFN γ and IL-12 during the development of immune responses to microbial pathogens, the cells responsible for initiating this interaction and the temporal sequence of the events by which they occur *in vivo* remained obscure.

In the current study, we generated novel mice displaying conditional deletion of the *Ifngr1* gene and used them to clarify the roles and cellular targets of IFN γ and IL-12 during initiation of protective immune responses against *Listeria monocytogenes*. *Ifngr1*^{ff} mice bred to *Vav-icre* mice were first analyzed to validate the effectiveness of our targeting and observed selective deletion of *Ifngr1* in hematopoietic cells. The resulting mice were highly susceptible to *Listeria* infection, a result that confirmed an earlier report that IFN γ responsiveness in hematopoietic cells was required to resolve systemic *Listeria*

infection (Yap and Sher, 1999). We then generated mice that lacked *Ifngr1* selectively in CD8 α^+ /CD103 $^+$ DCs by crossing our *Ifngr1^{ff}* mice to a specific line of *Itgax-cre* mice that preferentially drives cre expression in DCs. Using these mice I show that IFN γ acts on CD8 α^+ DCs to initiate the early production of IL-12 responsible for enhancing production of more IFN γ thereby establishing a pathway that controls the earliest stages of *Listeria* infection. Furthermore, *Ifngr1^{ff}* mice bred to *LysM-cre* which lacks IFN γ responsiveness in neutrophils and a subset of macrophages allowed us to identify neutrophils as one of the critical IFN γ -dependent effectors. Thus, our data identify CD8 α^+ DCs as both critical responders to IFN γ that is initially produced upon infection as well as the primary source of early IL-12 that is required to drive optimal IFN γ dependent anti-bacterial immune responses such as neutrophil activation during *Listeria* infection (Figure 36).

Our study shows that IL-12 production from CD8 α^+ DCs is transient and ceases 24 hours post-infection. The mechanism(s) underlying the transient nature of this initial IL-12 production is currently unknown. It is possible that infection of CD8 α^+ DCs results in their rapid elimination. Disappearance of CD8 α^+ DCs has been reported by others to occur at approximately 18 hours post-infection (Edelson et al., 2011). However, it is also possible that IL-12 production may be actively inhibited in these cells by IL-10 that may arise as a consequence of the infection. IL-10 is known to be a powerful inhibitor of IL-12 produced by dendritic cells (Huang et al., 2001). More work is needed to define the events that limit the initiation phase of the process.

Nevertheless, even though mice with IFN γ unresponsive CD8 α^+ DC show significant deficits in their capacity to produce IL-12 and display a highly compromised capacity to control the early stages of *Listeria* infection, they eventually control the infection. I show that Ly6C^{hi} inflammatory monocytes produce IL-12 that provides a mechanism to control the infection. The identification of inflammatory monocytes as an important source of IL-12 agrees with results of earlier studies (Kapadia et al., 2011; Tam and Wick, 2006; Zhan et al., 2010). Importantly, even though CD8 α^+ DCs, inflammatory monocytes, and even CD11b⁺ DCs have been identified as major sources of IL-12, the temporal aspects of their functions have, until now, not been investigated. Herein, it was shown that IL-12 production from CD8 α^+ DCs occurs first and requires the priming actions of IFN γ on these cells. This step is then followed by a shift of IL-12 production by inflammatory monocytes. As shown by others (Tam and Wick, 2006), one third of IL-12-producing Ly6C^{hi} inflammatory monocytes additionally produced TNF α and iNOS, thus identifying them as TipDCs (Serbina et al., 2003). IFN γ is required for the activation and maturation of TipDCs (Kang et al., 2008) and furthermore NK cell-produced IFN γ promotes the local differentiation of inflammatory monocytes into CD11c⁺MHCII⁺ DCs which serves as the major source of IL-12 during *T. gondii* infection (Goldszmid et al., 2012). During *Listeria* infection, Ly6C^{hi} monocytes may also need to be primed by IFN γ in order to optimally produce the second wave of IL-12. This possibility will be examined in future experiments once we breed IFN γ insensitivity selectively into these cells.

CD8 α^+ DCs play an obligate role in transporting *Listeria* into the PALS and thus are critical for establishing a productive infection (Edelson et al., 2011; Neuenhahn et al., 2006). Yet these same cells are also thought to be crucial for recruiting and activating innate cells in the foci of bacterial infection (Kang et al., 2008). It is therefore significant that at 12 hours, these foci contained CD8 α^+ DCs, IL-12, IFN γ^+ NK cells, neutrophils, and *Listeria* - essentially all the components needed to initiate the anti-*Listeria* response. While the selective abrogation of IFN γ responsiveness in CD8 α^+ DCs did not impede any spatial regulation of innate cell clustering and *Listeria* proliferation, I demonstrate that IFN γ responsiveness in CD8 α^+ DCs is important for reciprocal activation of CD8 α^+ DCs and NK/NKT cells. Although a kinetic analysis was conducted to understand the causal link between IFN γ production by NK/NKT cells and IL-12 production by CD8 α^+ DCs via flow cytometry, we are unable to determine which cytokine precedes the other. Similarly, *in situ* histologic approaches by Kang et al. revealed that IFN γ and IL-12 production coincided with each other at 12 hours post infection (Kang et al., 2008). When the levels of TNF α , IL-6, IFN γ , and IL-12 were monitored during *Listeria* infection, no obvious lag between onset of IL-12 and IFN γ could be detected *in vivo*, although production of TNF α was shown to precede the other cytokines (Liu and Kurlander, 1995; Nakane et al., 1992).

We showed previously that TNF α directly stimulates the release of IFN γ from NK cells (Tripp et al., 1993; Wherry et al., 1991), and it was demonstrated here that blockade of TNF α signaling inhibits the reciprocal activation of NK/NKT and CD8 α^+ DCs. Mice lacking both TNF receptors (TNFR1 and TNFR2) were also shown to be

deficient in IL-12 production, suggesting that TNF α plays a role in the eventual induction of IL-12 in response to *Listeria* infection (Zhan and Cheers, 1998). Thus, our findings suggest a model wherein TNF α potentiates the first wave of IFN γ from NK/NKT cells, which then induces CD8 α^+ DCs to produce IL-12 that stimulates NK/NKT cells to produce more IFN γ . Of note, our studies do not exclude a possible role of direct cellular interactions between NK/NKT cells and CD8 α^+ DCs. Functional interactions between NK cells and DCs are dependent upon both cell contact as well as cytokine production (Gerosa et al., 2002; Piccioli et al., 2002). Interestingly, Xu et al. reported that DCs stimulate IFN γ production from NK cells via triggering of TNFR2 on NK cells by transmembrane TNF α on DCs, instead of soluble TNF α (Xu et al., 2007). Our results support this concept because whereas we could inhibit early IFN γ and IL-12 production by mAbs that neutralize TNF receptors, we could not block them with our TN3-19.12 mAb (Sheehan et al., 1989) that potently neutralizes the soluble form of TNF α but not membrane-bound TNF α (data not shown). Thus, our data suggest that reciprocal activation between NK/NKT and CD8 α^+ DC is likely to occur in infection foci where DCs facilitate the clustering of NK cells via direct contact and cytokine secretion and are therefore consistent with the conclusion of a previous report by others (Kang et al., 2008).

The current study was possible because of the availability of the novel mice generated in this thesis that were engineered to display IFN γ unresponsiveness in specific tissues. Although mice with systemic IFNGR1 deficiency have been available for almost 20 years (Huang et al., 1993), the mice generated for this study are the first, to our knowledge, to display tissue specific deficiency of the *Ifngr1* gene. Recently, Kernbauer

et al. reported the use of mice with conditional deletion of the *Stat1* gene in DCs to study anti-*Listeria* responses (Kernbauer et al., 2012). Although STAT1 indeed plays a critical role in IFN γ R signaling, it is also required for IFN α/β R signaling. In *Listeriosis*, IFN γ is host protective while Type I IFNs are detrimental to the host (Carrero et al., 2006). Thus, the use of mice that lack the ability to respond to both classes of IFNs makes the interpretation of their results difficult.

In summary, the results of this thesis project indicate that IFN γ from NK/NKT cells directly potentiates IL-12 production from CD8 α^+ DCs. Considering that CD8 α^+ DCs transport *Listeria* and recruit innate cells such as NK/NKT cells into infection foci, the induction of IL-12 production by IFN γ appears to be both time- and location-specific. Thus, NK/NKT production of IFN γ and subsequent IL-12 production by CD8 α^+ DCs are critical initiators of the innate response to *Listeria* which lead to IFN γ -mediated activation of effect cells such as macrophages and neutrophils.

Future directions

Our identification of CD8 α^+ DCs and neutrophils as important targets of IFN γ provides the basis for future experiments using *Ifngr*^{ff} mice to further dissect the role of IFN γ signaling in different cell types during *Listeria* infection. Currently, *Ifngr*^{ff} mice have been bred with *CD4-cre*, *NKp46-cre*, *Foxp3-GFP-cre*, and *VE-cadherin-creER*^{T2} (Table 1). These mice will be also useful to study abilities to resist infection by other microbial pathogens such as *Leishmania major*, and several mycobacteria species, including *M. Bovis* and *M. avium* which require IFN γ responsiveness to be resolved

(Bach et al., 1997).

For example, IFN γ signaling in T cells skews the differentiation of naïve CD4 T cells into Th1 cells (Dighe et al., 1995; Macatonia et al., 1993). After breeding *Ifngr*^{ff} mice with *CD4-cre* mice which selectively delete *Ifngr* in T cells (Figure 37), we will explore the possibility that lack of IFN γ signaling in T cells skews the *in vivo* differentiation of naïve CD4⁺ T cells away from Th1 cells towards the other CD4⁺ T cell subsets i.e., Th2, Treg, or Th17 by a variety of immunological stimuli. Th2 and Treg cells are thought to deteriorate anti-bacterial and tumor responses by inhibiting cellular immunity. In contrast, Th17 cells are uniquely primed to mediate surveillance and early defense during infections by bacterial, mycobacterial, and fungal pathogen although Th17 cells have become notorious for their involvement in a range of autoimmune diseases (Stockinger and Veldhoen, 2007). In addition to affecting the induction of primary T cell response, IFN γ is also relevant to the contraction phase of T cell response (Badovinac et al., 2000). This study demonstrated that CD8⁺ T cells failed to contract in IFN γ -deficient mice after infection with an attenuated (*actA*-deficient) strain of *Listeria*. Furthermore, the absence of IL-12 receptor on antigen-specific T cells not only reduced the contraction, but also increased the generation of memory T cells against *Listeria* (Pearce and Shen, 2007). Thus, the reciprocal activation of CD8 α ⁺ DCs and NK/NKT cells inducing initial production of both IFN γ and IL-12 is likely to be crucial for regulating primary and memory T cell responses against bacterial infection.

Furthermore, the fact that these mice were generated on a pure C57BL/6 genetic background will make them widely useful for many different types of studies inducing

those where homogenous genetic backgrounds are a critical consideration such as tumor transplantation studies. We observed that IFN γ signaling is required for rejection of transplantable sarcomas (Dighe et al., 1994) and for prevention of primary tumor outgrowth (Kaplan et al., 1998). Previously we focused our efforts on defining the effects of IFN γ on the developing tumor itself and showed that IFN γ functions, at least in part, to enhance tumor cell immunogenicity by up-regulating its MHC class I antigen processing and presentation pathway (Shankaran et al., 2001; Strehl et al., 2005). However, our initial observations also pointed to an important role for IFN γ at the level of the host in controlling tumor outgrowth. Others also noted that host cell responsiveness to IFN γ is important for development of protective anti-tumor responses (Fallarino and Gajewski, 1999; Qin and Blankenstein, 2000; Zhang et al., 2008). However, since nearly all cells in the host express functional IFN γ receptors, it remains unclear which host cells need to respond to IFN γ in order to develop host protective tumor immunity. In fact, this question has led to considerable disagreement within the field as to the immune versus non-immune mechanisms underlying tumor rejection. Some have suggested that lymphocytes are the critical targets of IFN γ owing to its capacity to polarize the T cell response to Th1/CTL (Knutson and Disis, 2005) and to inhibit the capacity of regulatory T cells (Tregs) to establish an immunosuppressive tumor microenvironment (Sakaguchi et al., 2008). Others have suggested that IFN γ works to activate macrophages either inducing in them the capacity to nonspecifically kill tumor cells (Schreiber, 1984) or to form granulomas (Schuler et al., 2003) that restrict the access of carcinogens to normal cells. Still others have suggested that IFN γ inhibits angiogenesis either directly or indirectly via

inducing chemokines with potential angiostatic activity such as CXCL-9, -10, and -11 thereby causing tumor starvation and death (Qin et al., 2003). This argument has remained unresolved because it has not been possible to inactivate IFN γ responsiveness in individual tissues to define their specific roles in the rejection process. However, the recent results of bone marrow chimera experiments indicate that rejection of unedited MCA sarcomas requires IFN γ sensitivity at the level of both hematopoietic and non-hematopoietic host cellular compartments (Diamond et al., 2011). This observation has led us to form the hypothesis that immune elimination of developing tumors requires a coordinated multicellular response orchestrated by IFN γ 's effects on several different cellular populations including (but perhaps not limited to) regulatory T cells and endothelial cells.

For example, after breeding *Ifngr*^{f/f} mice with *Foxp3-cre* mice which will selectively delete *Ifngr* in regulatory T cells, we can test whether lacking IFN γ responsiveness in the Treg compartment should display heightened immunosuppressive capacity because the IFN γ produced during the anti-bacterial and tumor response cannot control Tregs. In collaboration with Dr. Hiroshi Shiku (Mie University, Japan), we identified that IFN γ production by CD8⁺ T cells abrogates the generation/activation of CD4⁺CD25⁺ Tregs in response to self-antigens that express Treg epitopes such as DNA J-like 2 (Nishikawa et al., 2005). Immunization of WT BALB/c mice with DNA encoding DNA J-like 2 resulted in enhanced pulmonary metastases following intravenous challenge with syngeneic CMS-5 tumor cells. This protocol also resulted in accelerated primary tumor development after MCA treatment due to the expansion of Treg in the

immunized hosts. When the plasmid encoding DNA J-like 2 was co-injected with one encoding a strong CD8⁺ T cell epitope (9m) that rapidly induces IFN γ , Treg were unable to suppress proliferating T cells and there was a marked reduction in pulmonary metastases and MCA-induced sarcoma formation. On the other hand, a role for IFN γ in maintaining Treg homeostasis and function has been noted by others using a model of anti-CD40 induced inflammation (Koch et al., 2009). This study could develop a contradictory prediction to the previously stated hypothesis—that introduction of IFN γ insensitivity into Tregs should reduce their function/homeostasis and thereby induce heightened anti-bacterial/ tumor potential in *Foxp3-cre⁺Ifngr^{ff}* mice.

The ability of IFN γ to inhibit tumor angiogenesis has been well documented by others and has also been confirmed in our MCA sarcoma transplantation model. However, to date no experiments have been forthcoming to distinguish between the possibilities that IFN γ -induced angiostasis causes tumor rejection or is merely a by-product of the rejection process. In addition, uncertainty remains as to the ultimate effectors of the angiostatic response. IFN γ has certainly been shown to have direct antiproliferative effects on endothelial cells (Ruegg et al., 1998). Some have proposed that production of IFN γ induced chemokines, specifically CXCL-9, -10 and/or -11, is a key event in the tumor rejection process due to their putative capacities to inhibit tumor angiogenesis (Dufour et al., 2002; Melter et al., 2001; Pertl et al., 2001; Sauty et al., 2001). To test whether this mechanism was relevant to MCA sarcoma rejection, our lab generated a hamster mAb (CXCR3-173) specific for murine CXCR3 that blocks chemokine binding *in vitro* and *in vivo* (Uppaluri et al., 2008). Using tumor transplantation approaches, we

could not show inhibitory activity of CXCR3-173 on rejection of over unedited MCA sarcomas (Uppaluri and Schreiber, unpublished). In follow-up studies we also found that unedited sarcomas were rejected by CXCR3^{-/-} mice in a manner similar to that observed with WT mice. Finally, we compared susceptibility to MCA tumorigenesis between WT and CXCR3^{-/-} mice and again noted no differences. Thus our data strongly suggest that tumor rejection is not related to the action of CXCR3 chemokines. Nevertheless, these results do not rule out a direct angiostatic role for IFN γ in the rejection process (Qin and Blankenstein, 2000). Taken together these data suggests that angiostasis may be a direct function of IFN γ produced during the anti-tumor immune response but still does not answer the question of whether this function leads to tumor rejection. For this reason, generating mice that lack the capacity to respond to IFN γ only in the endothelial compartment is extremely important and exciting. VE-cadherin-creER^{T2} can be used to get an endothelial cell specific deletion of *Ifngr1* (Monvoisin et al., 2006).

As shown above, the use of the *Ifngr1*^{ff} mice can be easily extended to explore the roles of IFN γ on the selected tissues/cell types in various bacterial infection models depending on which tissue-specific cre mice are chosen. Additionally, because the mice are on a homogeneous background they can be utilized in tumor immunology, autoimmunity models, and even transplantation model. By defining the specific targets of IFN γ we will obtain new levels of understanding of host responses to various immunological stimuli that may facilitate the development of new targeted treatments to direct the specificity of IFN γ -dependent immunological processes into controlling pathological diseases.

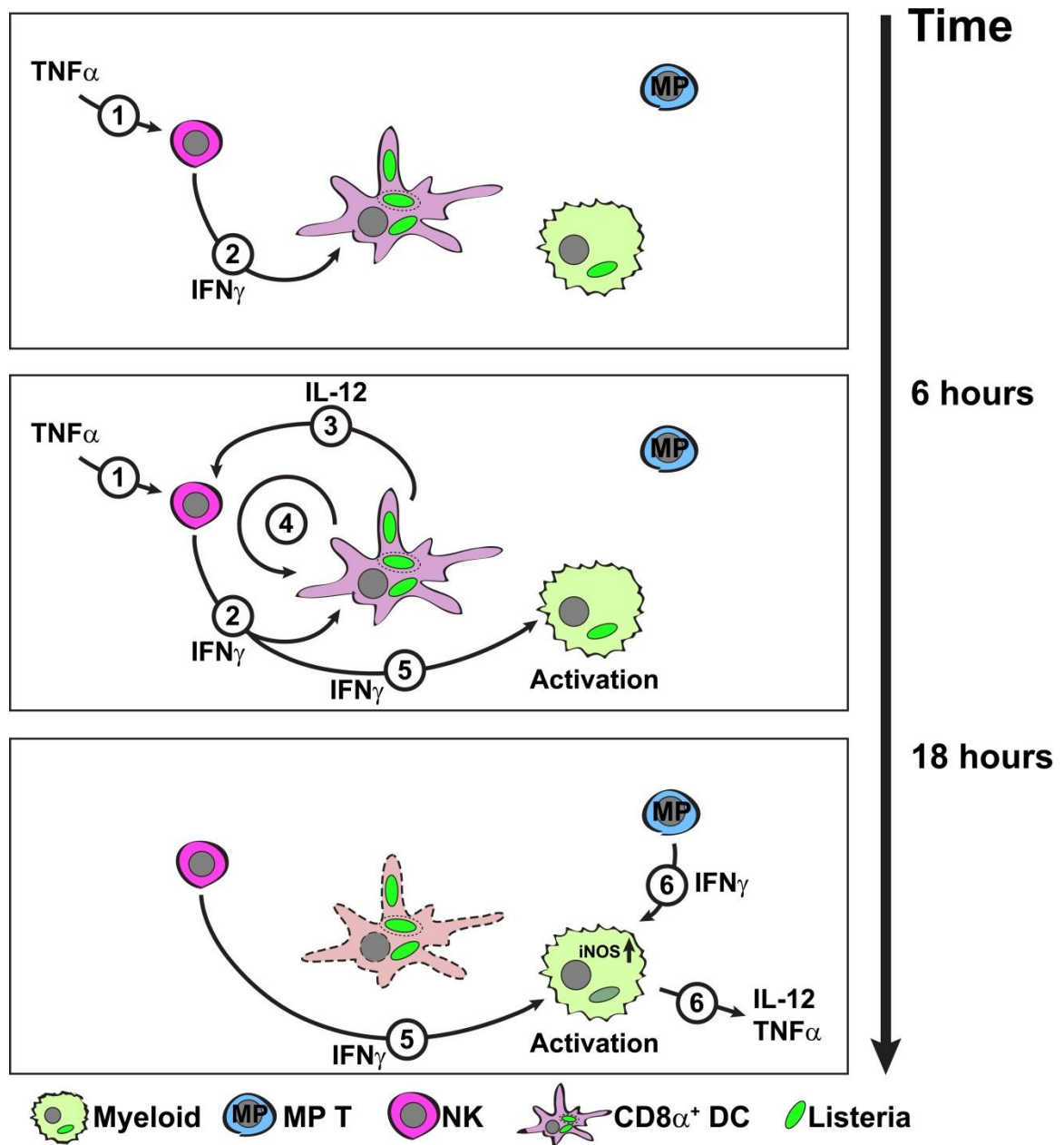


Figure 36. The proposed model for the initiating events of anti-*Listeria* responses.

Table 1: Current breeding status of mice with tissue-specific deletion of *Ifngr1*

| Tissue-specific <i>Ifngr1</i> KO mice | Tissue specificity | Current status |
|-----------------------------------------------------------------------|---------------------------|-----------------------|
| <i>Vav-icre</i> x <i>Ifngr1</i> ^{f/f} | All hematopoietic cells | Completed |
| <i>Itgax-cre</i> x <i>Ifngr1</i> ^{f/f} | Dendritic cells | Completed |
| <i>LysM-cre</i> x <i>Ifngr1</i> ^{f/f} | Macrophages, PMNs | Completed |
| <i>NKp46-cre</i> x <i>Ifngr1</i> ^{f/f} | NK/NKT cells | Completed |
| <i>CD4-cre</i> x <i>Ifngr1</i> ^{f/f} | T cells | completed |
| <i>VE-cadherin-creER</i> ^{T2} x <i>Ifngr1</i> ^{f/f} | Endothelial cells | completed |
| <i>Foxp3-GFP-cre</i> x <i>Ifngr1</i> ^{f/f} | Regulatory T cells | In process |

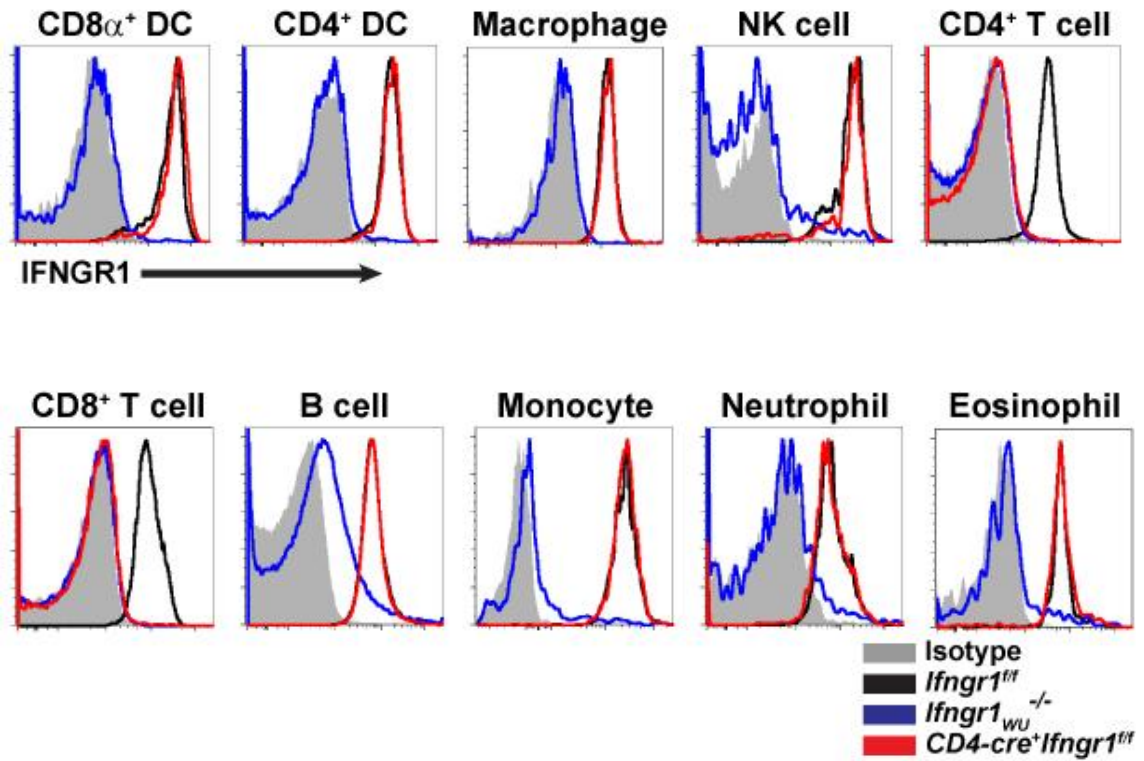


Figure 37. *CD4-cre*⁺*Ifngr1*^{f/f} has no IFNGR1 in T cells

IFNGR1 expression was measured using flow cytometry in *CD4-cre*⁺*Ifngr1*^{f/f} mice. The spleens and PBLs from *Ifngr1*^{f/f}, *CD4-cre*⁺*Ifngr1*^{f/f}, and *Ifngr1*_{WU}^{-/-} were harvested and stained with either biotinylated anti-IFNGR1 mAb or Isotype Ab with various lineage markers (Figure 1).

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