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Identifying the Initiating Events of Anti-*Listeria* Responses Using Mice with Conditional Loss of IFN- γ Receptor Subunit 1 (IFNGR1)

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Although IFN- γ is required for resolution of *Listeria monocytogenes* infection, the identities of the IFN- γ -responsive cells that initiate the process remain unclear. We addressed this question using novel mice with conditional loss of IFN- γ R (IFNGR1). *Itgax-cre⁺Ifngr1^{fl/fl}* mice with selective IFN- γ unresponsiveness in CD8 α^+ dendritic cells displayed increased susceptibility to infection. This phenotype was due to the inability of IFN- γ -unresponsive CD8 α^+ dendritic cells to produce the initial burst of IL-12 induced by IFN- γ from TNF- α -activated NK/NKT cells. The defect in early IL-12 production resulted in increased IL-4 production that established a myeloid cell environment favoring *Listeria* growth. Neutralization of IL-4 restored *Listeria* resistance in *Itgax-cre⁺Ifngr1^{fl/fl}* mice. We also found that *Itgax-cre⁺Ifngr1^{fl/fl}* mice survived infection with low-dose *Listeria* as the result of a second wave of IL-12 produced by Ly6C^{hi} monocytes. Thus, an IFN- γ -driven cascade involving CD8 α^+ dendritic cells and NK/NKT cells induces the rapid production of IL-12 that initiates the anti-*Listeria* response. *The Journal of Immunology*, 2013, 191: 4223–4234.

L*isteria monocytogenes* is an opportunistic pathogen that causes significant disease in neonates, the elderly, and immunocompromised individuals (1). Production of IFN- γ and cellular responsiveness to this cytokine in the host are crucial for the effective resolution of infection, as originally demonstrated using a neutralizing mAb to IFN- γ (2) and subsequently using mice lacking genes encoding IFN- γ (3); IFNGR1, the major ligand binding chain of the IFN- γ R (4); or STAT1, the major transcription factor that mediates IFN- γ R signaling (5). Other studies using SCID mice lacking T and B lymphocytes revealed that NK cells were a major source of IFN- γ early in the infection and that the IFN- γ produced by NK cells activated microbicidal activity in macrophages, thus providing the host with an ability to control the infection until such time that sterilizing adaptive immunity to the organism could develop (6–8). A deeper understanding of this in-

nate protective response to *Listeria* infection came when the cytokines TNF- α and IL-12 were found to play important roles in the induction of IFN- γ from NK cells (6–11). This work culminated in defining the feed-forward amplification process that leads to development of innate immunity not only to *L. monocytogenes* but also to many other intracellular pathogens (12).

However, despite all that is known about the importance of IFN- γ in the anti-*Listeria* response, the identities of the specific cellular targets of IFN- γ required for initiation of the response and effective control of the infection remain to be established. An early study used transgenic mice expressing a dominant-negative, truncated form of IFNGR1 in certain myeloid cell populations to show that myeloid cell responsiveness to IFN- γ was critical for promoting protective host responses to *L. monocytogenes* (13). Another study used radiation bone marrow chimera approaches to demonstrate that IFN- γ R expression in the hematopoietic compartment was required for controlling *Listeria* infection (14). However, because functional IFN- γ Rs are expressed in almost every host cell type (15), until now, it has not been possible to more precisely identify the key IFN- γ -responsive cells required to initiate the anti-*Listeria* response.

Recently, much attention has focused on the role of dendritic cells (DCs) in *Listeria* infection. DCs are the primary cell type that sense, ingest, and present exogenous Ags from pathogens to initiate the pathogen-specific adaptive-immune response (16). Within this population, the CD8 α^+ /CD103⁺ DC subsets were shown to play a major role in cross-presenting exogenous Ags to CD8⁺ T cells, thereby inducing host-protective cytotoxic T cell responses (17, 18). Recent studies using CD11c-DTR mice, in which the diphtheria toxin receptor was expressed only in CD11c⁺ cells, revealed that mice depleted of all DCs did not develop *Listeria* infection in the spleen (19, 20). Furthermore, using *Batf3*^{-/-} mice, which selectively lack CD8 α^+ /CD103⁺ DCs, a role was demonstrated for these specific DC subsets in establishing *Listeria* infection in the spleen and liver (21). Together, these findings support a scenario in which migratory CD8 α^+ DCs carry *L. monocytogenes*

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Abbreviations used in this article: DC, dendritic cell; ES, embryonic stem; IDT, Integrated DNA Technologies; MP, memory phenotype; PALS, periarteriolar lymphoid sheath; p.i., postinfection; qRT-PCR, quantitative RT-PCR.

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from their entry point in the splenic marginal zone to the periarteriolar lymphoid sheaths (PALS), where *L. monocytogenes* then multiply in the ensuing 12–24 h and establish an active infection (22, 23).

Although the aforementioned studies reveal a critical role for CD8 α ⁺/CD103⁺ DCs in *L. monocytogenes* transport and initiation of infection, they do not provide insight into the interactions of these cells with other immune cells and cytokines. Although the cross-presenting functions of CD8 α ⁺/CD103⁺ DCs are known to be influenced by type I IFNs (24), little is known about the functional effects of IFN- γ on these cells. Thus, we asked whether IFN- γ responsiveness in CD8 α ⁺/CD103⁺ DCs directly influenced their ability to initiate anti-*Listeria* responses. Therefore, we generated mice with a floxed *Ifngr1* gene (*Ifngr1*^{fl/fl} mice) on a C57BL/6 background and then bred them to either C57BL/6 *Vav-icre* or *Itgax-cre* mice to impart IFN- γ unresponsiveness either broadly in hematopoietic cells or specifically in the CD8 α ⁺/CD103⁺ DC subsets, respectively (25, 26). Using these novel mice, we report in this article the elucidation of the events that underlie development of the innate immune response to *L. monocytogenes* and show that IFN- γ responsiveness in CD8 α ⁺/CD103⁺ DCs plays a critical role in initiating this process.

Materials and Methods

Generation of *Ifngr1*^{fl/fl} mice

The TNLOX1-3–targeting vector (27) was used to generate the conditional *Ifngr1*-targeting construct and electroporated into B6/Blu embryonic stem (ES) cells (28). After selection, ES cells were analyzed for homologous recombination by PCR using a 5' external primer (f3: 5'-CCAGTGT-TTGCCCTTGGATCTG-3', Integrated DNA Technologies [IDT]) and a Neo primer (r3: 5'-GTTGGCTACCCGTGATATTGCTG-3', IDT). PCR-positive clones were verified by Southern blotting following BamHI digestion using 5' and 3' external probes (Fig. 1A). One ES cell clone displaying complete integration of the targeting construct was verified by PCR using loxP-specific primer sets (f1: 5'-AAACAGTAAACCCAGGGCTT-TGTAC-3'; r1: 5'-CAGCCTCTGAAATTCAAATGGC-3'; f2: 5'-GTGACGGGAGCACCTGTTAC-3'; r2: 5'-GTAAGTGCATTCATCTGGCCAG-3', IDT). The correctly targeted ES clone was expanded and transiently transfected with pTurbo-Cre (ES cell core; Washington University) for removal of the Neo resistance gene. Neo-sensitive clones were screened using 5' primers (5'-AAACAGTAAACCCAGGGCTTGTAC-3', IDT) and two 3' primers (5'-CAGCCTCTGAAATTCAAATGGC-3', 5'-CGTGGCACTGTAGATGTACTGTACAG-3', IDT) and confirmed by Southern blotting using the 5' probe. Neo-deleted subclones were injected into eight cell embryos using the laser-assisted microinjection technique (29).

Mice

B6 (C57BL/6NTac) mice were obtained from Taconic. *Itgax-cre* [007567; C57BL/6J-Tg (Itgax-cre, EGFP)4097Ach/J] mice (25) and *Vav-icre* [008610; B6.Cg-Tg(Vav1-cre)A2Kio/J] mice (26) were obtained from The Jackson Laboratory. Both *Itgax-cre* and *Vav-icre* mice were backcrossed onto the C57BL/6 background using speed congenic approaches (>99% purity; Rheumatic Diseases Core Center, Washington University) and then crossed to *Ifngr1*^{fl/fl} 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.

Infection

All mice were infected with indicated doses of *L. monocytogenes* (strain: EGD) in pyrogen-free saline. Colony counts were determined as previously described (30). In some experiments, portions of spleens and livers were fixed in 10% formalin, embedded in paraffin, and stained with H&E.

Ab treatment

To neutralize cytokines and/or block receptors, mice were treated i.p. on day -1 (relative to *Listeria* infection) with the following endotoxin-free preparations of mAbs: 200 μ g 11B11 mAb for IL-4 neutralization (31); 500 μ g SK113AE-4 mAb for IL-18 neutralization (32); 250 μ g each ALF-

161, B122, and JAMA-147 mAbs for IL-1 neutralization/blockade (33–35); 250 μ g each 55R-170 and TR75-54 mAbs for TNFR1 and TNFR2 blockade (36); and 250 μ g H22 mAb for IFN- γ neutralization (37). For in vivo depletion of NK/NKT cells, *Ifngr1*^{fl/fl} mice were treated with 200 μ g PK136 mAb (BioLegend) i.p. on days -2 and 0. Memory phenotype (MP) CD8⁺ T cells were depleted by injecting *Ifngr1*^{fl/fl} mice i.p. with 200 μ g CXCR3-173 mAb (38) on days -3 and 0.

Flow cytometry

The following mAbs were purchased from BioLegend and used as lineage markers: PerCP/Cy5.5 anti-CD3 ϵ (145-2C11), PE or allophycocyanin anti-NK1.1 (PK136), FITC or PE/Cy7 anti-CD4 (GK1.5), PerCP/Cy5.5 or allophycocyanin/Cy7 anti-CD8 α (53-6.7), FITC or PE anti-CD45R/B220 (RA3-6B2), allophycocyanin 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), allophycocyanin/Cy7 anti-Ly6G&C/Gr-1 (RB6-8C3), allophycocyanin/Cy7 anti-Ly6C (HK1.4), PerCP/Cy5.5 anti-Ly6G (1A8), FITC or allophycocyanin/Cy7 anti-CD11c (N418), PerCP/Cy5.5 anti-CD103 (2E7), allophycocyanin anti-CD183/CXCR3 (CXCR3-173), FITC anti-CD62L (MEL-14), PE anti-CD44 (IM7), and PE anti-CD31 (MEC13.3). PE anti-CD122 (TM- β 1), and PE anti-Siglec-F (E50-2440) were purchased from BD Biosciences. Allophycocyanin anti-Dec205 (205yekt) was purchased from eBioscience. Single-cell suspensions from various tissues were prepared and stained for IFNGR1 and pSTAT1 with biotinylated anti-IFNGR1 (GR20; BD Bioscience) and Alexa Fluor 647 anti-pSTAT1 (4a; BD Bioscience), as previously described (24). For intracellular cytokine staining, cells were stained with lineage markers without either in vitro restimulation 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/Cytoperm (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).

Quantitative real-time PCR

RNA was prepared from spleens and cell pellets of sorted cells using an RNeasy Mini/Micro Kit (QIAGEN). Purified RNA was reverse-transcribed to cDNA using random hexamers and Superscript III Reverse Transcriptase (Invitrogen). Quantitative real-time PCR reaction was carried out on a 7000 Sequence Detection System (Applied Biosystems) using Power SYBR Green PCR master mix (Applied Biosystems). Every real-time PCR was normalized by 18S. Specific primers (IDT) for quantitative real-time PCR (qRT-PCR) were designed using Primer Bank (<http://pga.mgh.harvard.edu/primerbank/>).

Immunohistochemistry

Alexa Fluor 488 anti-B220 (RA3-6B2), biotin anti-CD3 ϵ (145-2C11), and biotin anti-CD11b (M1/70) were purchased from BD Biosciences. Alexa Fluor 555-SA, Alexa Fluor 555 goat anti-rat IgG (H+L highly Cross-Adsorbed), and Alexa Fluor 647 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. Alexa Fluor 647 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 frozen sections were fixed in acetone at 4°C for 5 min. All slides were blocked with CAS Block (Invitrogen) and stained with the indicated Abs 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 pseudo-colored with SPOT RT camera software and merged with Adobe Photoshop.

Statistical analysis

The statistical analysis was performed using the Mann-Whitney *U* test and Prism software (GraphPad Software). Statistical *p* values \leq 0.05 were considered significant. Error bars indicate SEM.

Results

Generation and characterization of C57BL/6 *Ifngr1*^{fl/fl} mice

We generated a conditional knockout allele of *Ifngr1* in C57BL/6 ES cells by placing two loxP sites surrounding the third and fourth exons that encode the extracellular domain of IFNGR1 (Fig. 1A).

Deletion of this region results in a frame-shift mutation that induces expression of only a minimal portion of IFNGR1 that is unable to bind IFN- γ (39). Southern blotting and PCR analyses confirmed proper gene targeting (Fig. 1B, 1C). Flow cytometric analyses revealed that *Ifngr1^{fl/fl}* mice were indistinguishable from C57BL/6 mice on the basis of cell surface expression of IFNGR1 (Fig. 1D).

This same targeting also produced subclones of ES cells in which the entire floxed region was deleted when they were transfected with pTurbo-cre (Fig. 1A). The fully deleted subclones gave rise to C57BL/6 *Ifngr1^{-/-}* mice (Fig. 1E) that were named *Ifngr1^{wu}* to distinguish them from *Ifngr1^{-/-}* mice previously generated on a 129/SvEv background (4). Cells from *Ifngr1^{wu}* mice neither expressed IFNGR1 (Fig. 1D) nor responded to IFN- γ in vitro (as detected by STAT1 phosphorylation), but they responded normally to type I IFNs (Fig. 1F).

Generation of C57BL/6 mice with IFNGR1 deficiency either in all hematopoietic cells or selectively in CD8 α ⁺/CD103⁺ DCs

To delete *Ifngr1* in hematopoietic cells, we bred *Ifngr1^{fl/fl}* mice to *Vav-icre* mice (*Vav-icre⁺Ifngr1^{fl/fl}*) because the latter are known to

delete floxed genes in all hematopoietic cells (26). Hematopoietic cells from the resulting *Vav-icre⁺Ifngr1^{fl/fl}* mice neither expressed IFNGR1 (Fig. 2A) nor responded to IFN- γ treatment by phosphorylating STAT1 (Fig. 2B). In contrast, CD31⁺CD45⁻ endothelial cells from these mice displayed undiminished levels of IFNGR1 (Fig. 2A) and pSTAT1 after IFN- γ stimulation (Fig. 2B), thus demonstrating that IFNGR1 expression in the non-hematopoietic compartment was not affected in *Vav-icre⁺Ifngr1^{fl/fl}* mice.

Ifngr1^{fl/fl} mice were also bred to a particular strain of *Itgax-cre* mice (25) selected because we showed previously that they induce a selective deletion of floxed genes in CD8 α ⁺/CD103⁺ DCs (24). *Itgax-cre⁺Ifngr1^{fl/fl}* mice showed significant reductions in IFNGR1 expression primarily in splenic CD8 α ⁺ DCs, with a partial reduction in splenic CD4⁺ DCs and a slight reduction in macrophages (Fig. 3A). Splenic CD8 α ⁺ DCs and peripheral CD103⁺ DCs are functionally and developmentally related (40). Thus, 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^{fl/fl}* mice, whereas myeloid CD11b⁺ DCs from

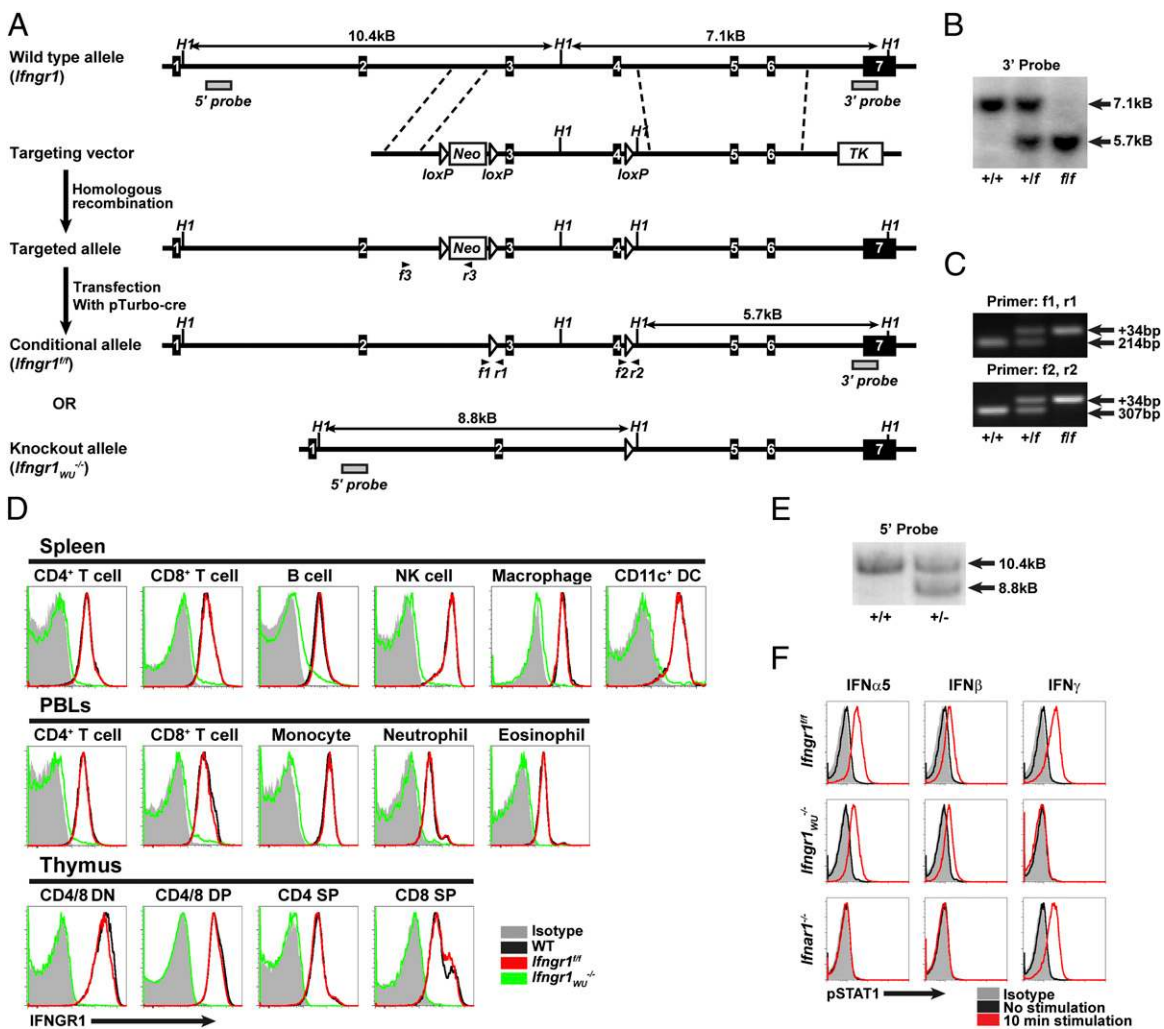


FIGURE 1. Generation of conditional IFN- γ R gene-targeted mice (*Ifngr1^{fl/fl}*). (A) Targeting strategy. Open gray boxes indicate external Southern probe to verify 5' and 3' homologous recombination. Small arrowheads indicate pairs of primers used in PCR to confirm either 5' homologous recombination (f3/r3) or insertion of loxP sites (f1/r1 and f2/r2). (B) Confirmation of germline transmission by examining 3' end of targeted allele by Southern blotting. (C) Verification of correct insertion of both 34-bp-long loxP sites by PCR. (D) Expression of IFNGR1 was assessed by flow cytometry analysis of spleen, peripheral blood leukocytes (PBLs), and thymus from wild-type, *Ifngr1^{fl/fl}*, and *Ifngr1^{wu}* mice. Data are representative of three separate experiments. (E) Confirmation of germline transmission of the *Ifngr1^{wu}* allele by 5' Southern blotting. (F) Measurement of phosphorylated STAT1 in *Ifngr1^{wu}* mice after in vitro IFN stimulation (10,000 U/ml IFN- α 5, 10,000 U/ml IFN- β , and 1,000 U/ml IFN- γ) for 10 min at 37°C. *f*, conditional allele; H1, BamH1; Neo, neomycin resistance gene; TK, HSV thymidine kinase gene; open triangle, loxP site; +, wild allele.

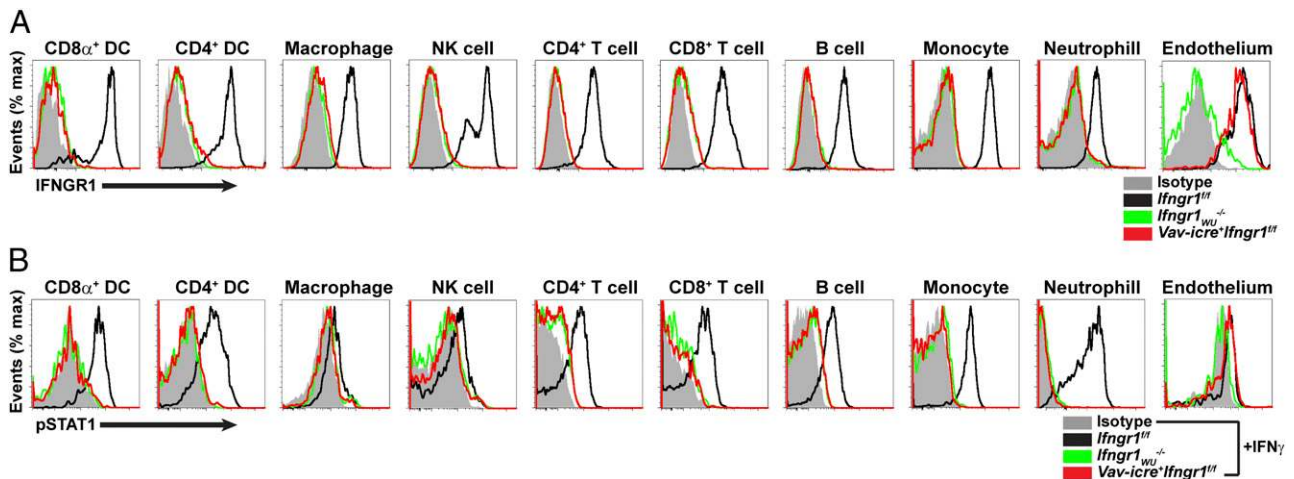


FIGURE 2. *Vav-icre*⁺*Ifngr1*^{fl/fl} mice lack functional IFNGR1 in hematopoietic cells. **(A)** Splenic IFNGR1 expression in *Vav-icre*⁺*Ifngr1*^{fl/fl} mice was measured. **(B)** After in vitro IFN- γ stimulation (1000 U/ml) for 15 min at 37°C, phosphorylated STAT1 in splenocytes from *Vav-icre*⁺*Ifngr1*^{fl/fl} mice was analyzed. All data are representative of at least two separate experiments. Gating strategies are depicted in Supplemental Fig. 1A.

these tissues did not (Fig. 3B). Of note, CD8 α ⁺ DCs from the *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice displayed significant response defects to IFN- γ , as evidenced by quantitating STAT1 phosphorylation (Fig. 3C) and CD40 upregulation (Fig. 3D). We observed the same response defects even using 10-fold higher doses of IFN- γ for a longer incubation time (Supplemental Fig. 1C). In contrast, no defect was observed in IFN- γ R signaling or responsiveness in CD4⁺ DCs, macrophages, and other hematopoietic cells. Thus, *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice display a highly specific IFN- γ unresponsiveness within the CD8 α ⁺/CD103⁺ DC compartment.

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

When challenged i.p. with two doses of *L. monocytogenes* (1×10^5 or 2.5×10^5), both *Vav-icre*⁺*Ifngr1*^{fl/fl} and *Ifngr1*^{w/w-/-} mice were more susceptible to infection than were *Ifngr1*^{fl/fl} mice, succumbing to infection by day 6 (Fig. 4A). Spleens and livers from *Vav-icre*⁺*Ifngr1*^{fl/fl} mice and *Ifngr1*^{w/w-/-} mice contained 10-fold more *L. monocytogenes* on day 1 and 100–1000-fold more bacteria on day 3 compared with the same organs from *Ifngr1*^{fl/fl} mice (Fig. 4B). These results functionally recapitulate the defect previously noted in *Ifngr1*^{-/-} bone marrow chimeras (14), revealing an obligate requirement for IFN- γ responsiveness in the hematopoietic compartment for resolution of *Listeria* infection.

IFN- γ responsiveness in CD8 α ⁺/CD103⁺ DCs is required for optimal anti-Listeria responses

When challenged i.p. with an LD₅₀ dose of *L. monocytogenes* (2.5×10^5), all of the *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice succumbed to infection (Fig. 4A, left panel). At a sublethal dose (1×10^5), *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice exhibited significantly increased *L. monocytogenes* burdens in the spleen, liver, and peritoneum compared with *Ifngr1*^{fl/fl} mice during the first 7 d of infection (Fig. 4C). As previously reported (41, 42), the LD₅₀ dose of *Listeria* administered i.v. (between 5×10^3 and 1×10^4) is much lower than that for i.p. challenge (2.5×10^5) (Fig. 4A, 4D). The defect in *Listeria* clearance in *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice was not dependent on the route of infection because *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice harbored more *L. monocytogenes* than did *Ifngr1*^{fl/fl} mice, even when the bacteria were administered i.v. (Fig. 4D, 4E). We failed to detect any bacteria in peritoneum after i.v. challenge. This result indicates that the larger *Listeria* burdens in the spleen and liver following i.p. infection compared with i.v. infection may represent an in-

creased unidirectional seeding of bacteria from the peritoneum in *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice to spleen/liver (Fig. 4C, 4E). Histologically, *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice showed highly increased numbers and sizes of *Listeria* foci in both spleen and liver compared with *Ifngr1*^{fl/fl} mice (Supplemental Fig. 2A). Eighteen hours after *Listeria* infection, infectious foci in the PALS were observed in both *Itgax-cre*⁺*Ifngr1*^{fl/fl} and *Ifngr1*^{fl/fl} mice, indicating that the normal migration of *Listeria*-infected CD8 α ⁺ DCs had occurred, regardless of their ability to respond to IFN- γ (Supplemental Fig. 2B). Mice solely expressing cre protein (*Vav-icre*⁺ or *Itgax-cre*⁺ mice) exhibited no differences in bacterial burdens compared with wild-type C56BL/6 mice, thus excluding potential influences of the cre protein on *Listeria* susceptibility (Supplemental Fig. 2C).

Two sets of data revealed that the increased susceptibility to *Listeria* infection in *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice was specifically due to IFN- γ unresponsiveness in CD8 α ⁺/CD103⁺ DCs. First, strong expression of EGFP was detected in CD8 α ⁺ DCs, less in CD4⁺ DCs, and not at all in other cells before and after *Listeria* infection (Supplemental Fig. 2D). This result rules out the possibility that *Listeria* infection might result in an infection-mediated deletion of *Ifngr1* in other cells by upregulating expression of the transgenic cre-EGFP bicistronic construct. Second, the observation that CD11c was not expressed in marginal zone macrophages and metallophilic marginal zone macrophages rules out the same possibility in these cells that trap *L. monocytogenes* at the onset of infection (43) (Supplemental Fig. 2E).

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

CD8 α ⁺ DCs and monocytes are implicated as the major cellular sources of IL-12 in *Listeria* infection (44–46). Therefore, we examined the effects of endogenously produced IFN- γ on IL-12 production by splenic CD8 α ⁺ DCs and monocytes from *Ifngr1*^{fl/fl} and *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice during the first 24 h of infection (Fig. 5). The limit of detection for ex vivo intracellular staining of IL-12 is 1×10^5 *L. monocytogenes* i.v., although we can still detect IL-12 transcripts with as few as 1×10^4 *L. monocytogenes* (Supplemental Fig. 3A, 3B). To synchronize bacterial infection and generate enough cytokine-producing cells, mice were infected i.v. with 10^6 *L. monocytogenes*. The percentage of splenic CD8 α ⁺ DCs producing IL-12p40 in *Ifngr1*^{fl/fl} mice increased to 4.2% at 9 h, reaching a maximum level of 9.4% at 12 h, then decreasing to 5.9% at 18 h, and back to baseline at 24 h (Fig. 5A, 5B). In contrast, the proportion

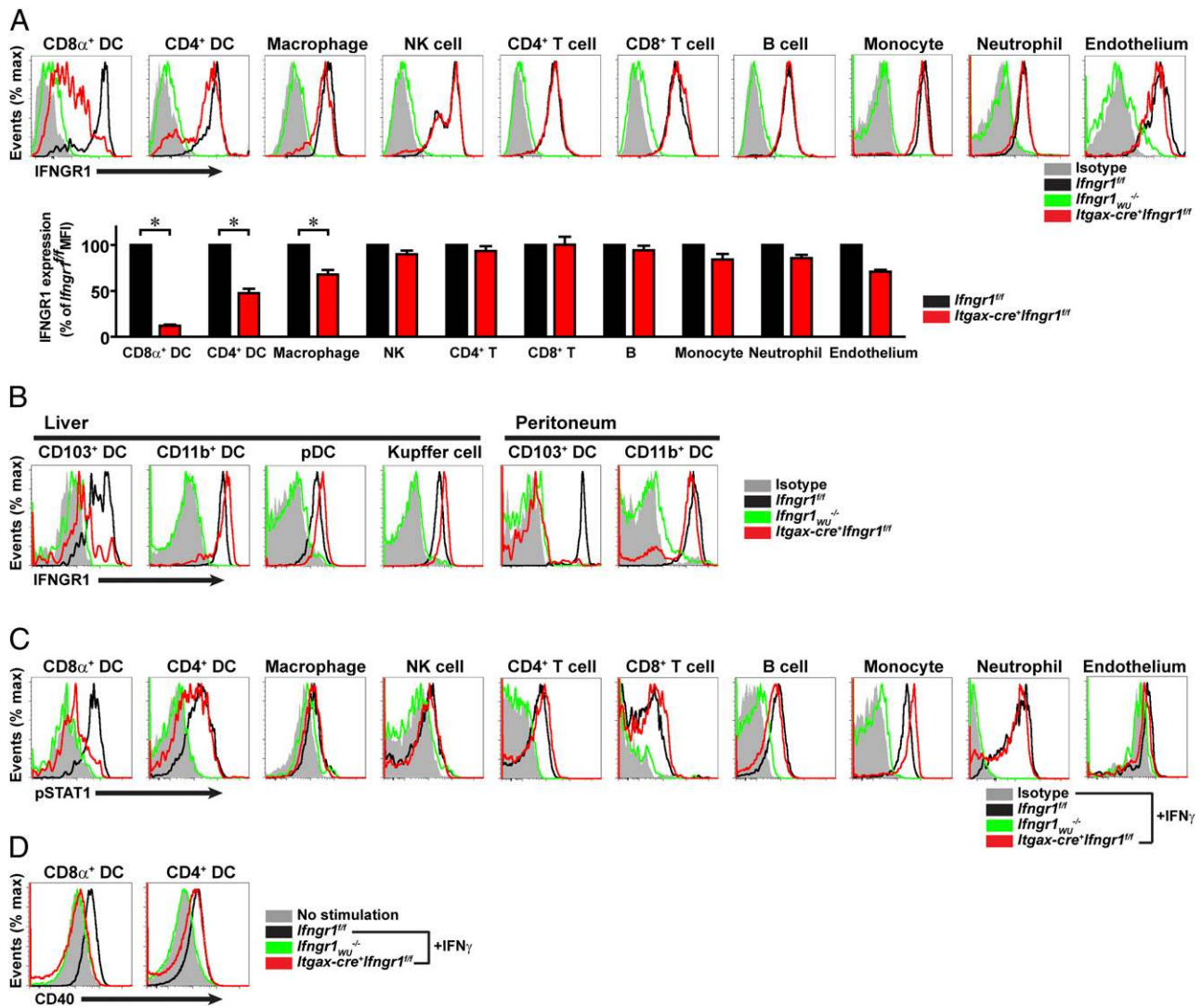


FIGURE 3. *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice lack functional IFNGR1 in CD8 α^+ /CD103 $^+$ DCs. **(A)** Splenic IFNGR1 expression in *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice was measured. IFNGR1 levels in the indicated cellular subsets in *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice compared with *Ifngr1*^{fl/fl} mice are summarized in the bar graph. **(B)** FACS analysis in liver and peritoneal cavity to confirm the lack of IFNGR1 expression in hepatic and peripheral CD103 $^+$ DCs in *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice. **(C)** After in vitro IFN- γ stimulation (1000 U/ml) for 15 min at 37°C, phosphorylated STAT1 in splenocytes from *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice was analyzed. pSTAT1 staining of unstimulated controls was indistinguishable from that in *Ifngr1*^{wu/wu} mice (Supplemental Fig. 1B). **(D)** Selective lack of upregulation of CD40 in splenic CD8 α^+ DCs in *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice after in vitro IFN- γ stimulation (500 U/ml) for 18 h at 37°C. Splenic CD11c $^+$ cells were positively enriched by MACS purification prior to IFN- γ stimulation. The wild-type, knockout, and isotype controls for (A) and (C) are the same as for Fig. 2A and 2B because the flow cytometry was performed at the same time. All data are representative of at least two separate experiments. Gating strategies are depicted in Supplemental Fig. 1A. * $p \leq 0.05$.

of IL-12p40-producing CD8 α^+ DCs from *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice was significantly less (1.9% at 9 h; 3.6% at 12 h; and 1.3% at 18 h). *Listeria*-infected *Ifngr1*^{wu/wu} mice showed complete abrogation of IL-12 production in CD8 α^+ DCs, demonstrating that the low-level induction of IL-12 in CD8 α^+ DCs from *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice was due to incomplete deletion of the *Ifngr1* gene (Fig. 5C). Expression of IL-12p35 was also significantly decreased in CD8 α^+ DCs from *Listeria*-infected *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice compared with infected *Ifngr1*^{fl/fl} mice, whereas CD8 α^+ DCs from either mouse did not produce significant amounts of IL-23p19 (Fig. 5D). Thus, CD8 α^+ DCs required IFN- γ responsiveness for IL-12 production.

IL-12p40 production by CD8 α^+ DCs subsided 24 h postinfection (p.i.). However, at this time, ~10% of Ly6C^{hi} monocytes stained positively for IL-12p40 in both *Ifngr1*^{fl/fl} and *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice (Fig. 5A, 5E). Approximately 30% of IL-12p40⁺Ly6C^{hi} monocytes had a phenotype ascribed to TNF/iNOS-producing DCs,

identified by their production of TNF- α and inducible NO synthase (Supplemental Fig. 3C). Thus, CD11c^{int}CD11b⁺Ly6C^{hi}Ly6G⁻ monocytes displayed a temporally delayed production of IL-12p40 compared with CD8 α^+ DCs.

IFN- γ insensitivity in CD8 α^+ DCs leads to a reduction in IFN- γ from NK/NKT cells

NK and NKT cells are major sources of IFN- γ during the early phases of *Listeria* infection, and IL-12 is known to be required for early IFN- γ production by NK cells (6). Therefore, we examined the consequences of IFN- γ unresponsiveness in CD8 α^+ DCs on the early induction of IFN- γ . The percentage of IFN- γ^+ NK/NKT cells in *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice was significantly decreased compared with that in *Ifngr1*^{fl/fl} mice at both 9 and 12 h p.i. (NK cells: 1.9% versus 4.8% at 9 h and 27.3% versus 36.5% at 12 h; NKT cells: 3.5% versus 8.0% at 9 h and 13.9% versus 21.1% at 12 h) (Fig. 6A–C). The reduced percentages of IFN- γ^+ NK/NKT cells from *Itgax-cre*⁺*Ifngr1*^{fl/fl}

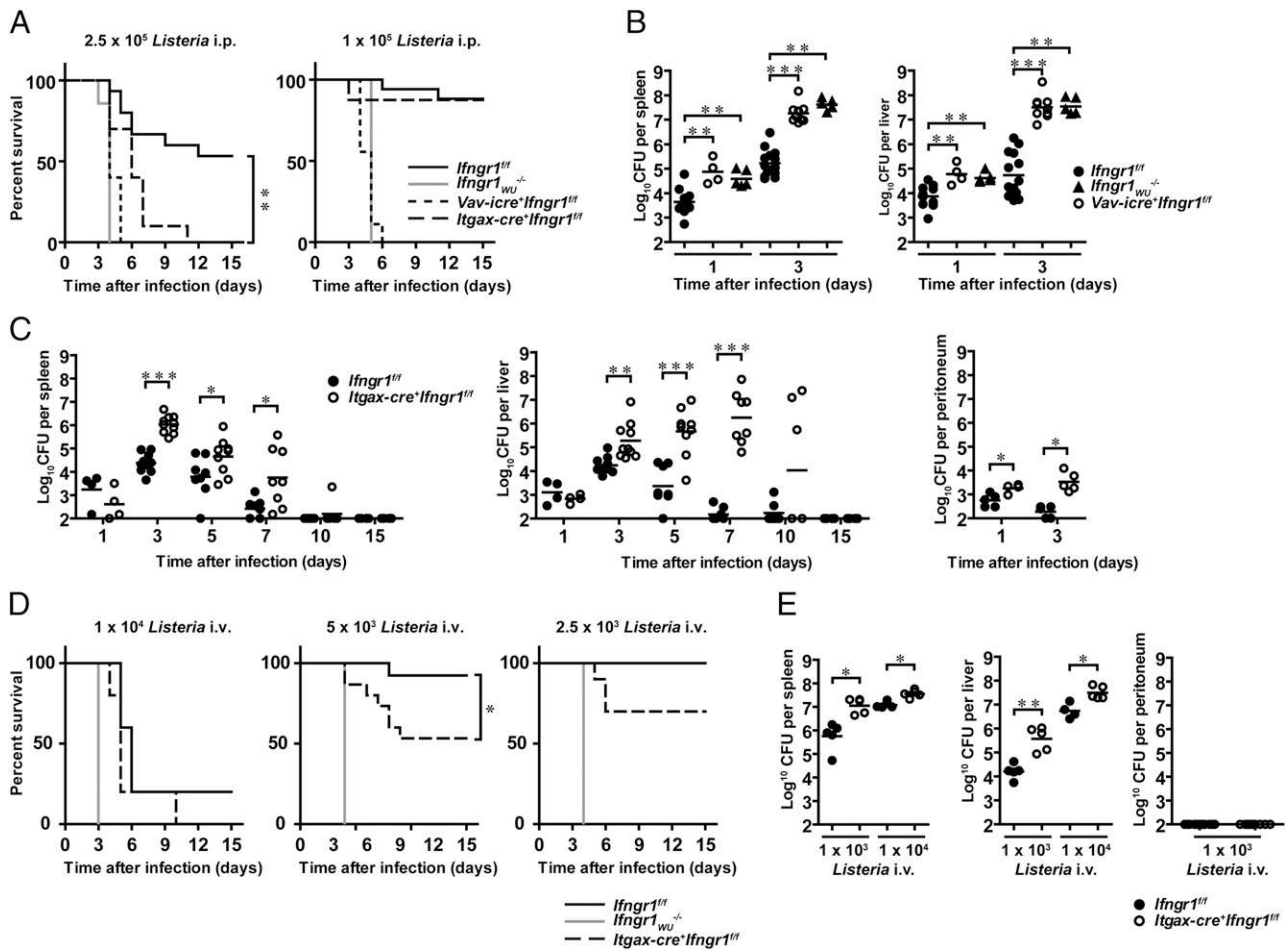


FIGURE 4. Both *Vav-icre*⁺*Ifngr1*^{fl/fl} and *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice display increased susceptibility to *Listeria* infection. **(A)** Mice were infected with 2.5×10^5 (left panel) or 1×10^5 (right panel) *L. monocytogenes* i.p., and the survival was monitored over time. **(B and C)** *Listeria* CFU in spleen, liver, and peritoneum infected with 10^5 *L. monocytogenes* i.p. **(D)** Mice were infected with 1×10^4 (left panel), 5×10^3 (middle panel), or 2.5×10^3 (right panel) *L. monocytogenes* i.v., and the survival was monitored over time. **(E)** *Listeria* CFU in spleen, liver, and peritoneum at 3 d after i.v. infection with 10^3 or 10^4 *L. monocytogenes*. Each symbol represents an individual mouse, and horizontal lines represent the mean \log_{10} CFU. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

mice occurred concomitantly with the decreased percentage of IL-12p40⁺CD8 α ⁺ DCs. However, the levels of IFN- γ ⁺ NK/NKT cells in *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice normalized to those found in *Ifngr1*^{fl/fl} mice by 18–24 h, when Ly6C^{hi} inflammatory monocytes produced a second wave of IL-12p40.

At 18 h p.i., IFN- γ production was observed in CD8⁺ T cells in both *Itgax-cre*⁺*Ifngr1*^{fl/fl} and *Ifngr1*^{fl/fl} mice (Fig. 6A, 6D). These T cells could represent the MP CD8⁺ T cells reported to produce IFN- γ during *Listeria* infection (47). MP CD8⁺ T cells express high levels of CD44 and CD62L and very high levels of CXCR3 and are selectively depleted by our CXCR3-specific mAb (CXCR3-173) (38) (Supplemental Fig. 4). CD4⁺ DCs, plasmacytoid DCs, CD4⁺ T cells, B cells, macrophages, and neutrophils were not significant sources of either IL-12 or IFN- γ in the first 24 h of infection (Supplemental Fig. 3D).

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

We considered the possibility that either NK/NKT cells or MP CD8⁺ T cells might be the initial source of IFN- γ that primes CD8 α ⁺ DCs for the first wave of IL-12. To test this idea, we treated *Ifngr1*^{fl/fl} mice with NK1.1 mAb, CXCR3-173 mAb, or both in combination (Fig. 7A) and assessed whether there was a corresponding decrease in IL-12 production by CD8 α ⁺ DCs (Fig. 7B). Depletion of NK and NKT cells resulted in a 53% decrease in

IL-12p40⁺CD8 α ⁺ DCs (2.7% anti-NK1.1 mAb-treated mice versus 5.7% control mice). In contrast, the elimination of ~80% of MP CD8⁺ T cells did not reduce IL-12p40⁺CD8 α ⁺ DCs. These results point to a cross-talk between NK/NKT cells and CD8 α ⁺ DCs mediated by IFN- γ and IL-12.

TNF- α induces IFN- γ from NK/NKT cells and thereby initiates the reciprocal activation of NK/NKT cells and CD8 α ⁺ DCs

The cytokines TNF- α , IL-18, and IL-1 are known to be involved in initiating anti-*Listeria* immunity, in part by inducing IFN- γ production from NK cells (8, 35, 48). Therefore, we assessed whether any of these cytokines initiated the cross-talk between NK/NKT cells and CD8 α ⁺ DCs. *Ifngr1*^{fl/fl} mice were treated with mAbs that either neutralize specific cytokines or block their receptors prior to infection (Fig. 8). Dual blockade of TNFR1 and TNFR2 decreased the percentage of IFN- γ ⁺ NK cells from 6 to 3.2% (47% decrease) (Fig. 8A) and of NKT cells from 11 to 2.8% (75% decrease) (Fig. 8B), which was accompanied by a reduction in IL-12p40⁺CD8 α ⁺ DCs from 6.6 to 2.6% (61% decrease) (Fig. 8C). As expected, direct neutralization of IFN- γ achieved a comparable 64% decrease in IL-12p40⁺CD8 α ⁺ DCs (Fig. 8C). In contrast, IL-18 neutralization had no effect on IL-12p40 production, although neutralizing IL-18 in combination with blocking TNFR1 and TNFR2 downregulated IFN- γ ⁺ NK cells 20% more than did dual-TNFR blockade alone (Fig. 8A). Neutralization of IL-1 caused

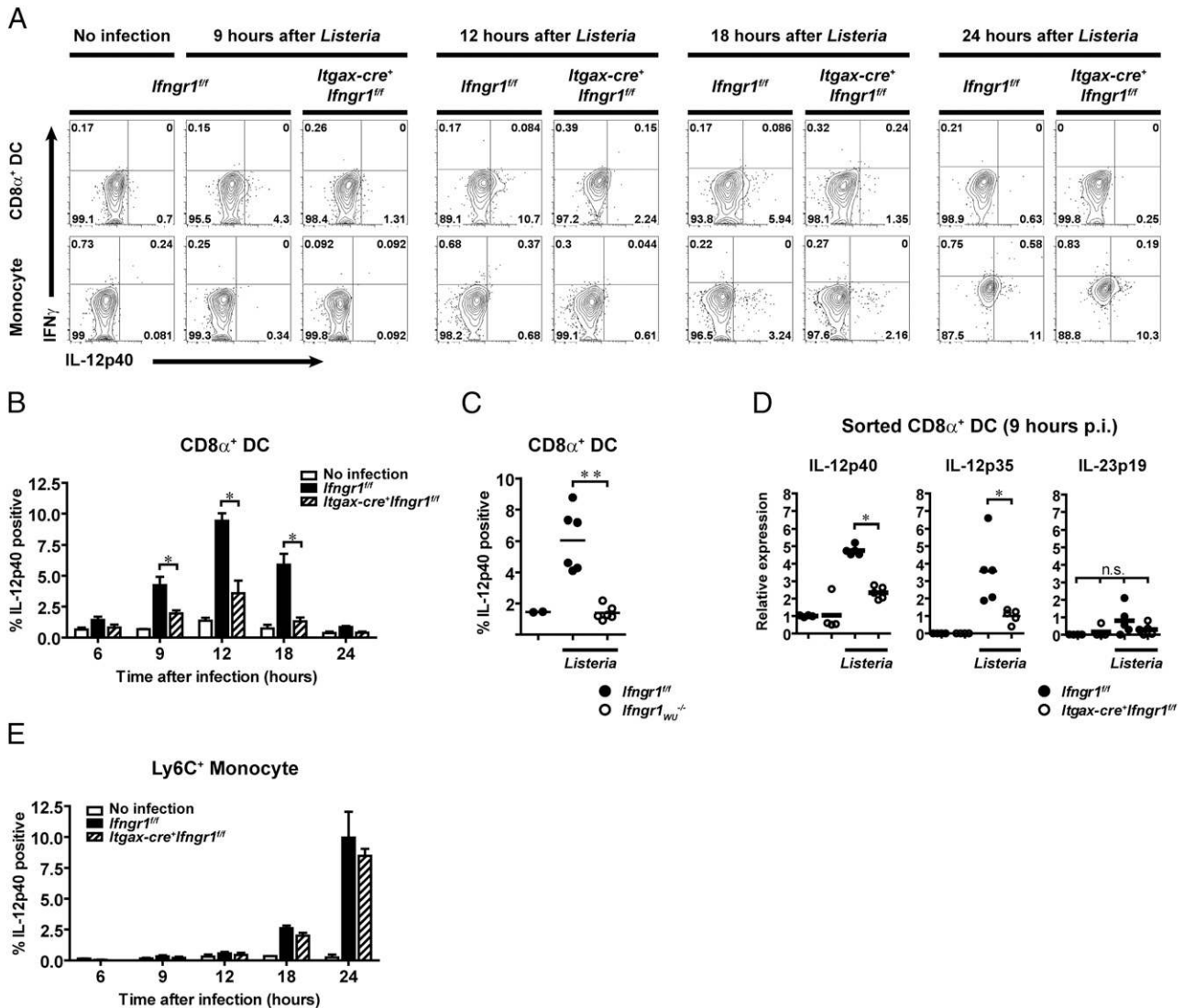


FIGURE 5. Early production of IL-12 from CD8α⁺ DCs is significantly decreased in *Listeria*-infected *Itgax-cre⁺Ifngr1^{fl/fl}* mice. All mice were infected with 10⁶ *L. monocytogenes* i.v. (A) Representative flow cytometry plots for IL-12p40 expression from CD8α⁺ DCs and Ly6C^{hi} monocytes. For each quadrant, gating was based on cells from uninfected controls that were analyzed at every time point. For simplicity, only the uninfected control at 9 h p.i. is shown. (B) Summary of percentages of IL-12p40⁺CD8α⁺ DCs in the spleen during the first 24 h of infection (n ≥ 4 at each time point). (C) Percentages of IL-12p40⁺CD8α⁺ DCs in the spleen from *Ifngr1^{fl/fl}* mice and *Ifngr1^{wu/-}* mice at 9 h of infection. (D) The expression of indicated genes in sorted CD8α⁺ DCs from spleens was determined by qRT-PCR after 9 h of infection. (E) Summary of percentages of IL-12p40⁺Ly6C^{hi} monocytes in the spleen during the first 24 h of infection (n ≥ 4 at each time point). *p ≤ 0.05, **p ≤ 0.01. n.s., not significant.

a 24% decrease in IL-12p40⁺CD8α⁺ DCs without altering the percentage of IFN-γ⁺ NK/NKT cells (Fig. 8C). Thus, the initial production of IFN-γ from NK/NKT cells upon *Listeria* infection that sets in motion a reciprocal activation of NK/NKT cells and CD8α⁺ DCs is a consequence of a process induced predominantly by TNF-α with potential participation of other early arising proinflammatory cytokines.

IL-4 production increases the Listeria susceptibility of Itgax-cre⁺Ifngr1^{fl/fl} mice by inducing alternatively activated macrophages

Enhanced *Listeria* susceptibility of *Itgax-cre⁺Ifngr1^{fl/fl}* mice could have resulted from a loss-of-function of the early IL-12/IFN-γ-amplification cycle or from a gain-of-function process due to inappropriate induction of cytokines that create a *Listeria*-permissive environment. Thus, we compared systemic cytokine mRNA induction in spleens of infected *Itgax-cre⁺Ifngr1^{fl/fl}* mice and *Ifngr1^{fl/fl}* mice (Fig. 9A). As expected, at 9 h p.i., splenocytes from

Itgax-cre⁺Ifngr1^{fl/fl} mice exhibited a significantly decreased transcription of IL-12p40, IL-12p35, and IFN-γ compared with *Ifngr1^{fl/fl}* mice. In contrast, we observed enhanced induction of IL-4 transcripts selectively in *Itgax-cre⁺Ifngr1^{fl/fl}* mice. IL-10 and IL-13 transcripts were induced comparably in both *Itgax-cre⁺Ifngr1^{fl/fl}* mice and *Ifngr1^{fl/fl}* mice. NKT cells were identified as one source of IL-4 in *Itgax-cre⁺Ifngr1^{fl/fl}* mice (Fig. 9B). We next examined whether increased *Listeria* susceptibility in *Itgax-cre⁺Ifngr1^{fl/fl}* mice was due to increased IL-4 production. Administration of IL-4-neutralizing mAb (11B11) to *Itgax-cre⁺Ifngr1^{fl/fl}* mice significantly reduced their *Listeria* burden, bringing bacterial loads down to levels observed in *Ifngr1^{fl/fl}* mice (Fig. 9C). IL-4 neutralization in *Ifngr1^{fl/fl}* mice slightly increased the ability of these mice to resist infection, but this increase was not statistically significant. These results demonstrate that the increased bacterial burdens seen in *Itgax-cre⁺Ifngr1^{fl/fl}* mice represented a gain-of-function process resulting from the ectopic expression of IL-4 as a consequence of the absence of early IL-12 from CD8α⁺ DCs.

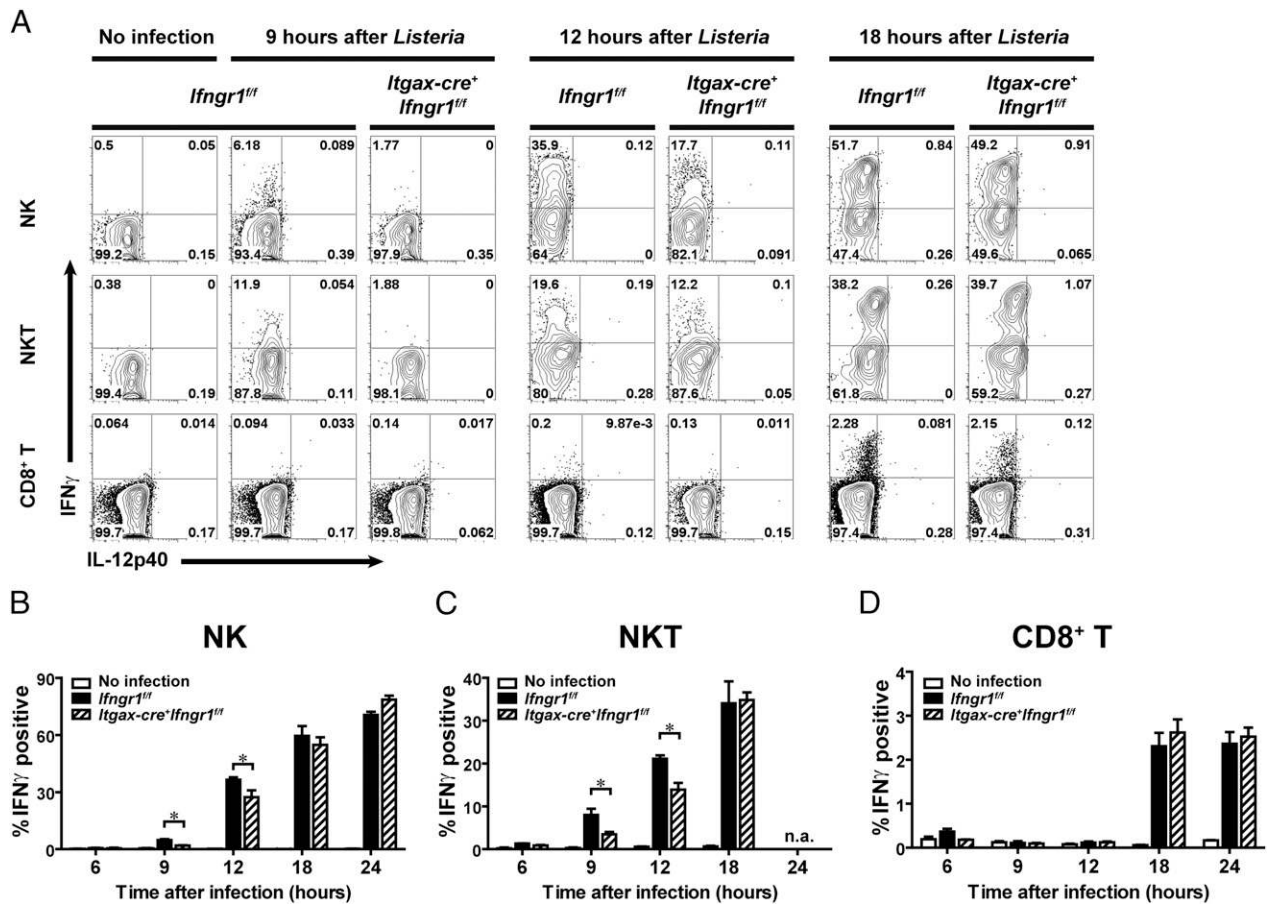
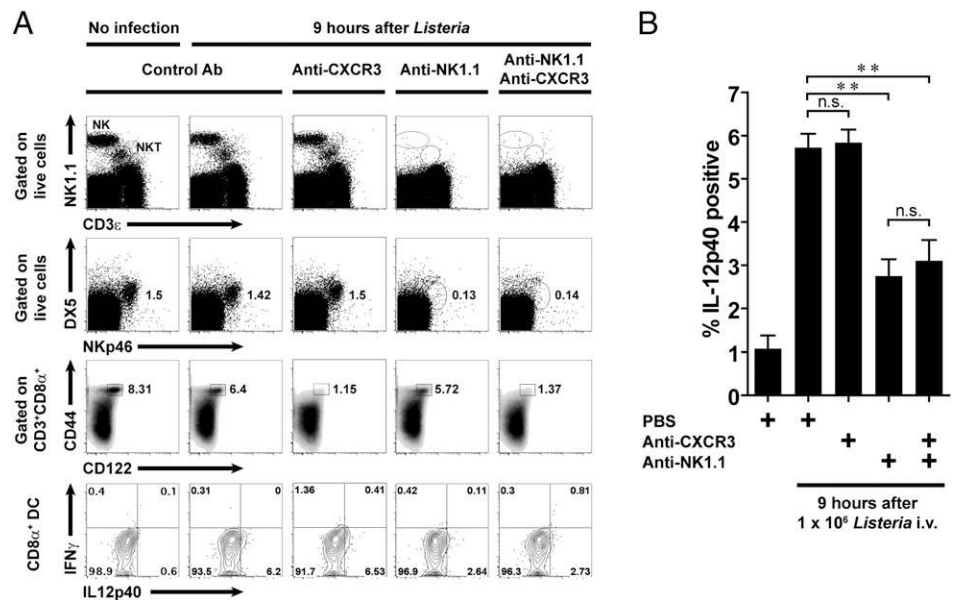


FIGURE 6. Early production of IFN- γ from NK and NKT cells is significantly decreased in *Listeria*-infected *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice. All mice were infected with 10⁶ *L. monocytogenes* i.v. (A) Representative plots for IFN- γ from NK, NKT, and CD8⁺ T cells during the first 24 h of infection. Summary of the percentages of IFN- γ ⁺ splenic NK cells (B), NKT cells (C), and CD8⁺ T cells (D), respectively ($n \geq 4$ at each time point). * $p \leq 0.05$.

IL-4 is known to induce alternatively activated (M2) macrophages that do not possess strong bactericidal activity compared with IFN- γ -activated (M1) macrophages (49). Strikingly, at 12 h p.i., splenocytes from *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice expressed significantly higher levels of mRNA encoding arginase 1, mannose receptor, and the secretory protein Ym1—hallmarks of M2 macrophages—

compared with *Ifngr1*^{fl/fl} mice (Fig. 9D). Moreover, expression of inducible NO synthase—a hallmark of M1 macrophages—was decreased in *Itgax-cre*⁺*Ifngr1*^{fl/fl} mice compared with *Ifngr1*^{fl/fl} mice. Thus, these results demonstrate that the lack of IFN- γ responsiveness in the CD8 α ⁺ DC compartment not only compromises the initiation of the anti-*Listeria* response (i.e., reduces the early

FIGURE 7. Depletion of NK/NKT cells producing early IFN- γ significantly reduces IL-12p40 production from CD8 α ⁺ DCs. (A) Representative FACS plots documenting depletion of NK/NKT cells by anti-NK1.1 mAb (top and second rows), depletion of MP CD8⁺ T cells by anti-CXCR3 mAb (third row), and IL-12p40 production from CD8 α ⁺ DCs at 9 h of infection with 10⁶ *L. monocytogenes* i.v. (bottom row). (B) Summary of percentages of IL-12p40⁺ CD8 α ⁺ DCs after treatment with depleting mAbs ($n \geq 5$). ** $p \leq 0.01$. n.s., not significant.



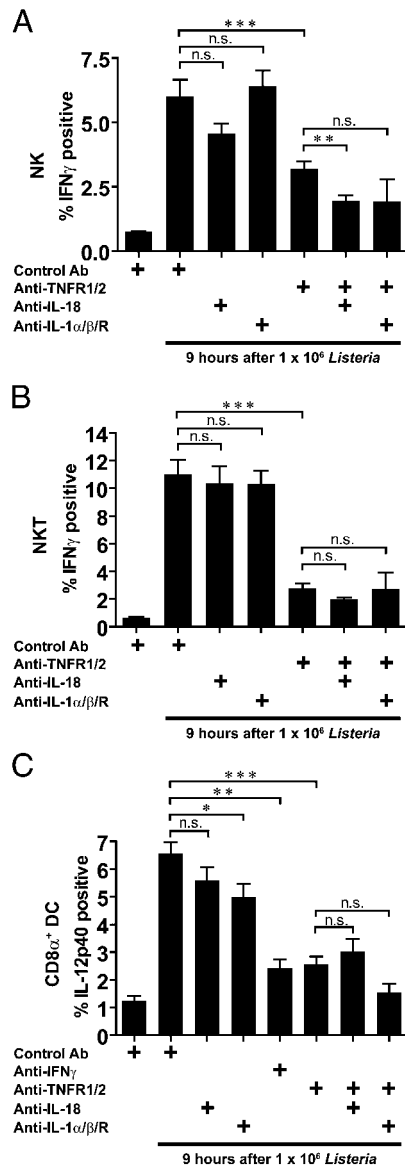


FIGURE 8. Blockade of TNF- α prior to *Listeria* infection reduces both IL-12 production from CD8 α^+ DCs and IFN- γ production from NK/NKT cells. *Ifngr1^{fl/fl}* mice were treated with indicated mAbs (as described in *Materials and Methods*) prior to infection with 10^6 *L. monocytogenes* i.v. Spleens were harvested at 9 h p.i., and cells were analyzed for expression of IL-12p40 and IFN- γ by intracellular cytokine staining. Percentages of IFN- γ^+ NK cells (**A**), IFN- γ^+ NKT cells (**B**), and IL-12p40 $^+$ CD8 α^+ DCs (**C**) are plotted as bar graphs ($n \geq 4$). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. n.s., not significant.

induction of IL-12, IFN- γ , and M1 macrophages), it also allows for expression of IL-4 that effects early polarization of the macrophage compartment to alternatively activated M2 macrophages that lack the critical effector functions needed to destroy *L. monocytogenes*.

Discussion

Several years of studies established that the innate anti-*Listeria* response begins within the first 12 h p.i. (12). This response is characterized by the presence of a number of cytokines—the three studied here—TNF- α , IFN- γ and IL-12—as well as type 1 IFNs, IL-1 α /IL-1 β , IL-6, IL-10, and others. This early response also involves various cells: tissue-resident macrophages, DCs, neu-

trophils, $\gamma\delta$ T cells, NK cells, and innate CD8 $^+$ T cells. In this study, we used novel mice with a selective deficit of IFNGR1 expression in CD8 α^+ DCs to identify the roles of TNF- α , IFN- γ , and IL-12 in initiating the critical cytokine and cellular interactions that lead to the effective elimination of *Listeria* infection.

TNF- α , potentially produced by infected splenic marginal zone macrophages (50), sits at the top of the initiating cytokine cascade and induces the first IFN- γ from NK/NKT cells that, in turn, induces the early IL-12 from CD8 α^+ DCs. TNF- α was originally identified as a major participant with IL-12 in the induction of IFN- γ by NK cells, although the sequence of events was never defined (7). The transient IFN- γ -dependent induction of IL-12 from CD8 α^+ DCs represents a key step in forming an amplification loop that enhances IFN- γ by NK/NKT cells, depresses the latter's ability to produce IL-4, and establishes an environment within the myeloid compartment that remains receptive to stimulation of enhanced antimicrobial function. Despite the defect in early IL-12 production within several hours p.i., the biggest difference in spleen colony counts was observed in *Itgax-cre⁺Ifngr1^{fl/fl}* mice at day 3 after *Listeria* infection. It was reported that administration of either rIL-12 or anti-IL-12 Ab into *Listeria*-infected mice produced effects on bacterial counts after day 3 (51). This timing agrees with ours and supports the conclusion that defects in early IL-12 production require a certain time window before they manifest changes in bacterial burdens. The basis of the transient nature of the initial IL-12 production remains unclear. Disappearance of CD8 α^+ DCs was reported to occur at ~ 18 h p.i. (21). It is also possible that IL-12 production may be actively inhibited by IL-10, a powerful inhibitor of IL-12 produced by DCs (52). It is noteworthy that there is production of IL-10 in splenocytes in the first 9 h of infection. Strikingly the cellular source of IL-12 shifts, in a relatively short period of time, from CD8 α^+ DCs to Ly6C $^{\text{hi}}$ inflammatory monocytes, whose vastly greater numbers induce higher levels of IFN- γ from NK/NKT cells.

The role of NK1.1 $^+$ cells in host defense against *Listeria* has been seemingly controversial. We (6–8, 10) and other investigators (9, 11) found that IFN- γ produced by NK cells plays an important role during *Listeria* infection by activating mononuclear phagocytes. In contrast, it was proposed that NK1.1 $^+$ cells have a detrimental role in listeriosis by studies showing enhanced clearance of *Listeria* after mAb depletion of NK1.1 $^+$ cells (53, 54) or in $\text{J}\alpha 18^{-/-}$ mice lacking the majority of NKT cells (55). This apparent discrepancy can be explained by one particularly novel finding made in this study using *Itgax-cre⁺Ifngr1^{fl/fl}* mice showing that the first wave of IFN- γ /IL-12 from NK/NKT cells and CD8 α^+ DCs contributes to the clearance of *Listeria* infection mainly by controlling IL-4 production from NKT cells (and possibly other cells, such as basophils, eosinophils, mast cells, and innate lymphoid type 2 cells) (56). A rapid burst of IL-4 from splenic NKT cells was previously observed in C57BL/6 mice, which peaked at 30 min p.i. and disappeared by 3 h (57), and neutralization of this transient IL-4 resulted in increased *L. monocytogenes* resistance in C57BL/6 mice (58). Thus, depletion of IL-4-producing NKT cells by anti-NK1.1 mAb treatment or genetic deficiency of NKT cells possibly rendered host more resistant to infection, whereas the loss of IFN- γ -producing NK/NKT cells is rapidly compensated for by IFN- γ -producing MP T cells. A recent study using adoptive transfer of NK and MP T cells into IFN- $\gamma^{-/-}$ mice showed that MP T cells colocalize with *Listeria* and macrophages, whereas NK cells do not, thus providing the latter with a spatial advantage in mediating IFN- γ -dependent clearance of *Listeria* (59). Our data support a model wherein early IL-12 production from CD8 α^+ DCs not only promotes IFN- γ production from NK/NKT cells, but also suppresses IL-4 production from NKT cells. It is not known

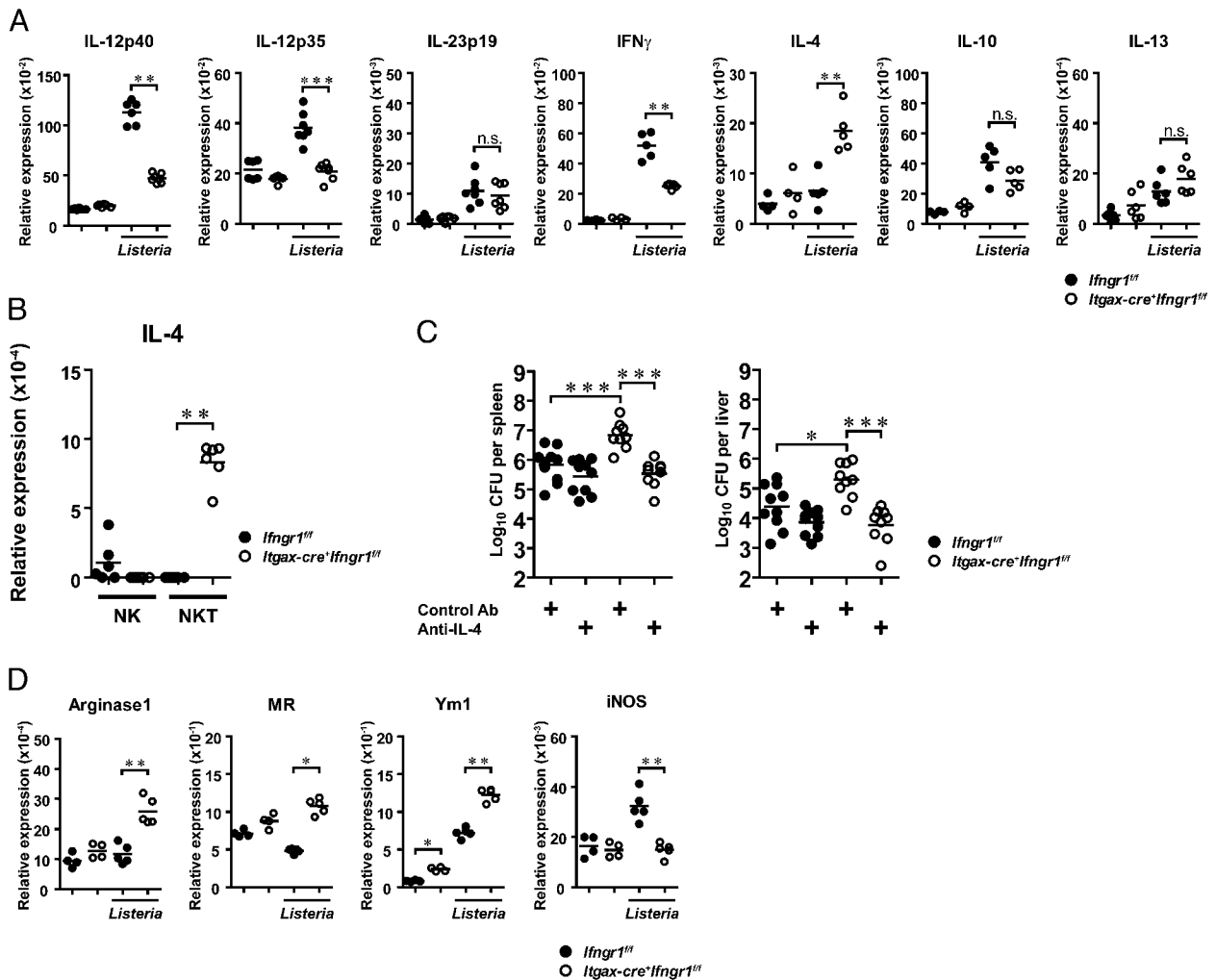


FIGURE 9. Neutralization of IL-4 restores *Listeria* resistance in *Itgax-cre⁺Ifngr1^{fl/fl}* mice. **(A)** Spleens were harvested at 9 h p.i. with 10^6 *L. monocytogenes* i.v. and analyzed for the expression of indicated genes by qRT-PCR. **(B)** IL-4 expression in NK and NKT cells sorted from spleens was determined by qRT-PCR after 9 h of infection with 10^6 *L. monocytogenes* i.v. **(C)** *Listeria* CFU in spleen and liver of infected mice at 3 d p.i. Mice were treated with 11B11 mAb prior to infection with 10^3 *L. monocytogenes* i.v. Data are a combination of two separate experiments. **(D)** The expression of indicated genes was analyzed by qRT-PCR at 12 h p.i. with 10^6 *L. monocytogenes* i.v. All qRT-PCR data are represented relative to the expression of 18S (ΔC_t). To facilitate visualization, values were transformed as indicated on the y-axis. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

whether the second wave of IL-12 produced by Ly6C^{hi} monocytes also participates in downregulating IL-4 production.

An issue to note is that the interactions between NK cells and DCs are dependent upon both cell contact and cytokine production (60, 61). Interestingly, Xu et al. (62) reported that IFN- γ production from NK cells is stimulated via triggering of TNFR2 on NK cells via membrane-associated TNF- α but not via soluble TNF α . Our results support this concept because, although we could inhibit early IFN- γ and IL-12 production using mAbs that block TNFRs, we could not inhibit early IFN- γ production using our TN3-19.12 mAb (63) that neutralizes soluble forms, but not membrane-associated forms, of TNF- α (data not shown). Thus, we suggest that reciprocal activation between NK/NKT and CD8 α^+ DCs is likely to occur in infectious foci containing both cell types via direct cellular contact, as well as cytokine secretion (20).

Thus, our studies and those of other investigators indicate at least two distinct functions for CD8 α^+ DCs in the innate immune response to *L. monocytogenes*. First, CD8 α^+ DCs play an obligate role in transporting *L. monocytogenes* into the PALS and, thus, are required for establishing a productive infection (19, 21). Second, as shown in this study, CD8 α^+ DCs play a critical role in producing

the first IL-12 that initiates an IFN- γ - and IL-12-dependent amplification loop. The selective abrogation of IFN- γ responsiveness in CD8 α^+ DCs does not impede *L. monocytogenes* transport, spatial regulation of innate cell clustering, or *L. monocytogenes* proliferation. This result demonstrates that the two critical functions of CD8 α^+ DCs are largely independent of one another.

In sum, this study reveals that NK/NKT cell production of IFN- γ and subsequent IL-12 production by CD8 α^+ DCs are critical initiators of the innate response to *L. monocytogenes* and, thus, illustrate how genetically homogeneous mice with tissue-selective defects in IFN- γ responsiveness help to refine our understanding of IFN- γ 's physiologic roles in vivo.

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Disclosures

The authors have no financial conflicts of interest.

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