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**Priming of Natural Killer Cells by Nonmucosal Mononuclear
Phagocytes Requires Instructive Signals from Commensal
Microbiota**

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Permissive signals from the commensal microbiota are required for infection-induced type I interferon gene expression by non-mucosal mononuclear phagocytes

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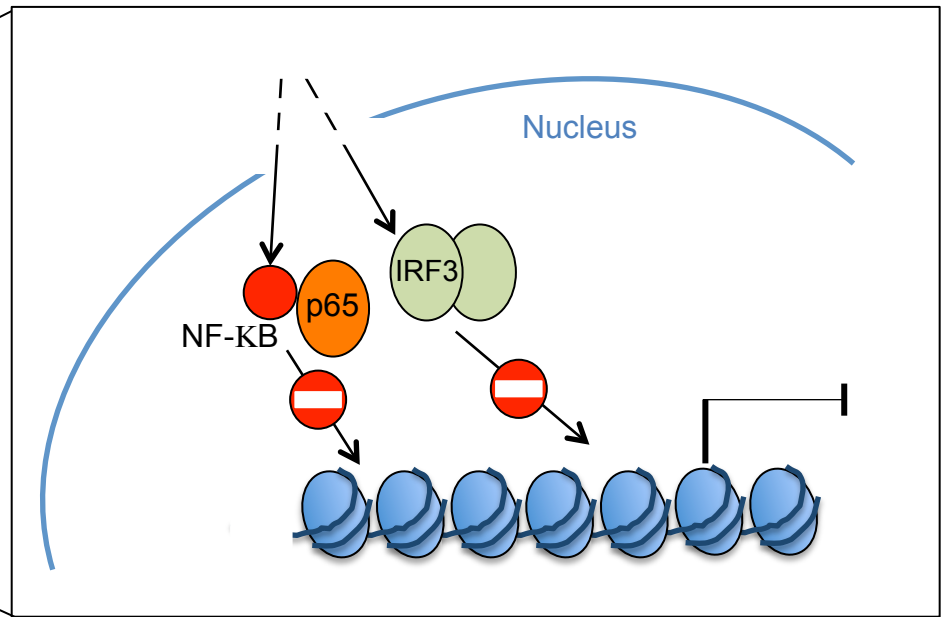
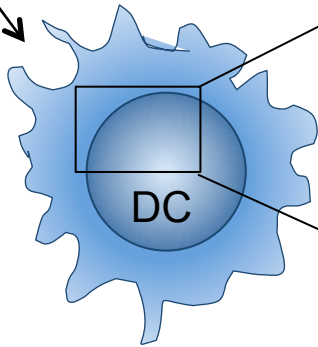
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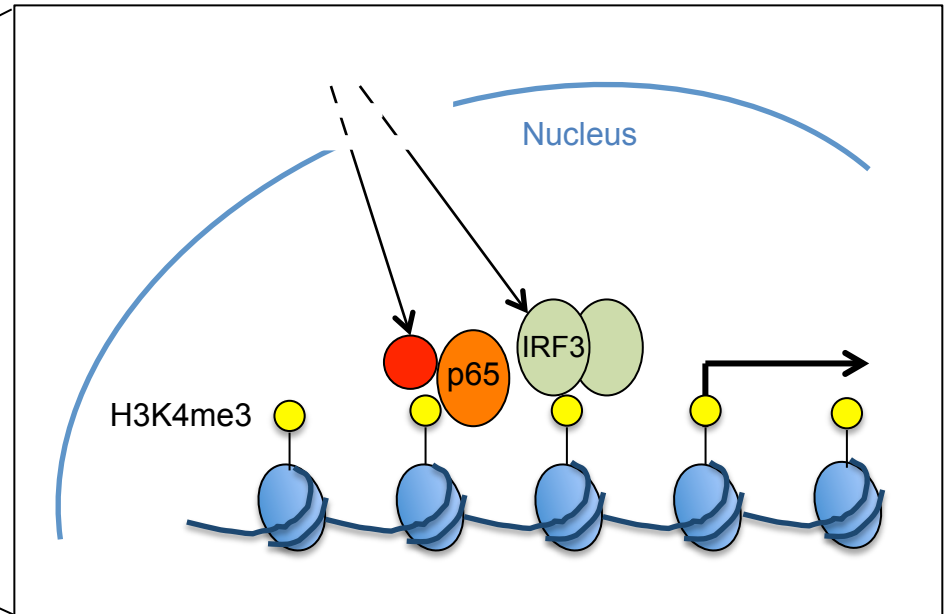
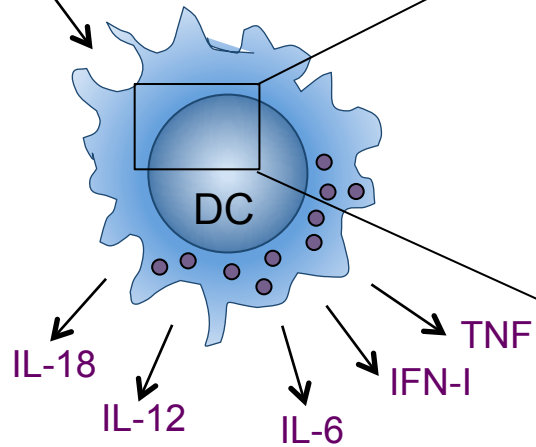
Germ-free

virus,
bacteria



Conventional

virus,
bacteria



Abstract

Mononuclear phagocytes are an important component of an innate immune system perceived as a system ready to react upon encounter of pathogens. Here, we show that mononuclear phagocytes residing in non-mucosal lymphoid organs of germ-free mice failed to produce type I interferons (IFN-I) in response to microbial stimulation. Consequently, NK cell priming and anti-viral immunity were severely compromised. Pathogen-inducible expression of a set of inflammatory response genes, including the various IFN-I genes, required poising by signals from the commensal microbiota. Whereas signaling downstream of various pattern recognition receptors and nuclear translocation of the transcription factors NF- κ B p65 and IRF3 were normal in mononuclear phagocytes of germ-free mice, binding to their respective cytokine promoters was impaired, which correlated with the absence of activating histone marks. Our data reveal a previously unrecognized role for postnatally colonizing microbiota in the introduction of chromatin level changes in the mononuclear phagocyte system, thereby poising expression of central inflammatory genes to initiate a powerful systemic immune response during viral infection.

Highlights

- Impaired function of non-mucosal NK cells from germ-free mice
- Mononuclear phagocytes of germ-free mice fail to produce IFN-I
- Germ-free mice are susceptible to systemic viral infections
- Microbiota poises IFN-I gene expression at the level of chromatin

Introduction

Mucosal surfaces of multicellular organisms are constantly exposed to a complex ecosystem of commensal bacteria. It is believed that millions of years of co-evolution have led to a mutualistic relationship between microbes and host cells (Hooper and Macpherson, 2010; Round and Mazmanian, 2009). The indigenous microbiota is now considered to be an important factor in shaping mucosal immunity and initiating immune signaling to maintain epithelial homeostasis (Artis, 2008; Hill and Artis, 2010). Although most studies have focused on the role of the intestinal microbiota, it was recently shown that respiratory tract microbiota regulates mucosal immunity to influenza A virus infection (Ichinohe et al., 2011). Collectively, current knowledge strongly supports the view that immune function at mucosal sites is controlled by indigenous microbiota.

In contrast to the instrumental role of indigenous microbiota in immune cell development and function at mucosal surfaces, the broader role of microbiota in shaping systemic immunity and immunity at non-mucosal sites is largely unexplored. However, this issue is highly significant in light of epidemiological and clinical data describing a marked increase of several immune-mediated disorders in “Western” lifestyle populations (Bach, 2002; Noverr and Huffnagle, 2004). Some of these increasingly prevalent diseases (i.e., type 1 diabetes and rheumatoid arthritis) occur at non-mucosal sites and are associated with deviated T helper cell responses. The rate at which these diseases have increased is too fast to be explained by genetic alterations. Therefore, it has been suggested that environmental factors, such as a reduced incidence of childhood infections or perturbations in the composition of the commensal microbiota, may be an important contributing factor (Bach, 2002; Noverr and Huffnagle, 2004) which is supported by data from germ-free and gnotobiotic mice (Mazmanian et al., 2005; Wen et al., 2008; Wu et al., 2010).

It is highly unlikely that T helper cells directly integrate signals from the commensal microbiota, but the underlying, microbe-induced alterations of the innate immune system are largely unknown. Cellular constituents of the mononuclear phagocyte system (i.e., dendritic cells (DC), macrophages) are equipped with various pattern recognition receptors allowing them to sense microbes. Mononuclear phagocytes then engulf microbes and gain antimicrobial activity but also inducibly express co-stimulatory molecules and soluble factors required to prime and coordinate adaptive immunity (Janeway, 1989). In non-mucosal lymphoid organs (such as spleen and peripheral lymph nodes), the total numbers of macrophages (Bauer et al., 1963; Morland et al., 1979; Shelton et al., 1970) and both migratory and resident DC subpopulations are not affected in germ-free mice (Walton et al., 2006; Wilson et al., 2008). However, several reports indicate that anti-microbial activity of macrophages from germ-free mice may be reduced (Mitsuyama et al., 1986; Morland et al., 1979). This is in line with a recent study showing the requirement of commensal-derived peptidoglycan in educating bone-marrow resident neutrophils for efficient killing of bacterial pathogens (Clarke et al., 2010).

In addition to being phagocytic and antigen-presenting cells, mononuclear phagocytes produce cytokines and chemokines required for coordinated and effective adaptive immune responses to viral and bacterial pathogens. Viral components are sensed by TLR, NLR (NOD-like receptors) or nucleotide sensors such as RNA helicases, the signaling pathways of which converge on the serine phosphorylation of interferon regulatory factors (IRF)3 and 7. Activated IRF3 and IRF7 induce the transcription of IFN-I (Tamura et al., 2008). IFN-I are a central requirement for immune responses to viral infections (Kawai and Akira, 2006) and act through binding to the IFN- α and β receptor (IFNAR) expressed by virtually all nucleated cells. IFNAR signaling induces an antiviral effector program within infected and uninfected cells (Tamura et al., 2008). In addition, IFN-I-activated DC induce cell-mediated immunity by

NK cells and CD8 T cells mainly through the induction of IL-15 expression and its trans-presentation by DC to lymphoid cells (Lucas et al., 2007; McCartney et al., 2009). The role of commensal microbiota in the production of IFN-I is unclear. While some studies suggested unimpaired IFN-I responses after infection of germ-free animals with viruses or bacteria *in vivo*, others found reduced IFN-I production when splenocytes from germ-free mice were stimulated with virus-infected cells (De Somer and Billiau, 1966; Ito et al., 1976).

NK cell activity is controlled by IFN-I at various levels. In IFNAR-deficient mice, NK cell function cannot be elicited after infection with viruses or bacteria or after injection of purified microbial ligands (Lucas et al., 2007; McCartney et al., 2009). In contrast to long-held dogma, NK cells are not naturally active killers but rather need to be rendered fully functional in a process referred to as NK cell “priming” (Fehniger et al., 2007; Kang et al., 2008; Lucas et al., 2007). NK cells can be primed by IFN-I-activated DC that trans-present IL-15, a cytokine that serves as a central priming signal (Degli-Esposti and Smyth, 2005; Lucas et al., 2007; Mortier et al., 2008). The impact of the commensal microbiota on NK cell activity is unknown. Early studies in germ-free animals report either small (Herberman et al., 1975) or no differences (Huh et al., 1981) in NK cell-mediated cytotoxicity. However, these analyses were performed in the absence of priming signals and unprimed NK cells in general show only minor cytotoxic activity (Lucas et al., 2007).

Here, we show that NK cells residing in non-mucosal lymphoid organs of germ-free mice could not be primed to mount effective anti-viral immunity. Adoptive transfer experiments revealed that this is not an NK cell-intrinsic defect but rather reflects impaired priming of NK cells by mononuclear phagocytes. Macrophages and DC from germ-free mice failed to produce IFN-I in response to microbial ligands or viral infection. Pattern recognition receptor-initiated signals led to unimpaired activation of NF- κ B and IRF3 in mononuclear

phagocytes of germ-free mice, but their binding to the promoter regions of various inflammatory response genes (e.g., *Ifnb1*, *Tnf*, *Il6*) was diminished. Trimethylation of lysine 4 of histone protein 3 (H3K4me3), a histone mark characteristic of transcriptionally active genes, constitutively marked the promoters of most inflammatory response genes in mononuclear phagocytes from conventional mice but not germ-free mice. Collectively, our data demonstrate a previously unappreciated role for the commensal microbiota in calibrating the function of mononuclear phagocytes at non-mucosal sites, likely by introduction of chromatin-level changes to poise expression of a set of genes required for potent immunity to infections.

Results

The commensal microbiota is required for effective priming of NK cells

We analyzed phenotype and function of NK cells residing in non-mucosal organs of germ-free (GF) mice or mice housed under conventional conditions (specific pathogen-free, SPF). We probed representation, phenotype and maturation of NK cells in the spleen of germ-free mice and found no major deviations in relative (Figure 1A) or absolute (Figure 1B) NK cell numbers. NK cell maturation was analyzed by determining the fraction of NK cells expressing KLRG1 (Huntington et al., 2007) or those co-expressing CD11b and CD27 (Hayakawa and Smyth, 2006). KLRG1 expression by NK cells was not reduced in germ-free mice (Figure 1C). While co-expression of CD11b and CD27 was not significantly different between NK cells from germ-free and SPF mice, the fraction of immature CD27⁺CD11b⁻ NK cells was reproducibly increased in germ-free animals (Figure 1D). Phenotypic characterization of NK cells from germ-free and SPF mice did not reveal any major differences (Figures 1E and 1F). Similar to NK cells from SPF mice, NK cells from germ-free mice uniformly co-expressed the IL-2/15R β chain (CD122), the integrin very late antigen 2

(CD49b or DX5) and activating NK cell receptors such as NKG2D and NKp46 (Figure 1E). In addition, NKG2A and inhibitory and activating Ly49 receptors, including the Ly49H receptor, were expressed by comparable subsets of NK cells from germ-free and SPF mice (Figure 1F). Thus, commensal bacteria are not required for the development, differentiation and maturation of splenic NK cells.

Next, we primed NK cell function by injecting various purified microbial ligands that signal via distinct pattern recognition receptor pathways into germ-free mice. Splenic NK cells were then analyzed for their *ex vivo* activity against the standard NK target cell line YAC-1. Despite the fact that NK cells appeared phenotypically normal in germ-free mice, microbial stimulation did not lead to priming of splenic NK cell effector functions (Figures 1G-I and S1A). Similar results were obtained when NK cell activity was primed by injecting CD40 antibodies to directly trigger mononuclear phagocytes (Figures 1H and 1I). While granzyme B expression by NK cells could not be induced after injection of poly(I:C) into germ-free mice (Figures 1G and S1A), expression of dynamin 2, a protein involved in granule exocytosis (Arneson et al., 2008), was microbiota-independent (Figure S1B). In addition, NK cells isolated from poly(I:C)-injected germ-free mice could not be activated for IFN- γ production when activating receptors (i.e., NKG2D, Ly49D) were directly crosslinked *in vitro* (Figures S1C and S1D). We also tested whether NK cell functionality in SPF mice could be reverted after *per os* treatment with a mix of antibiotics. The treatment led to the absence of any culturable aerobic bacteria and a 10^5 - 10^6 -fold reduction in colonic anaerobic bacteria (Figure S2A). After 10 days of antibiotic treatment, priming of NK cells for cell-mediated cytotoxicity (Figure 2A) and cytokine production (Figure 2B) was significantly impaired.

NK cells are required for immunity against mouse cytomegalovirus (MCMV) infection in C57BL/6 mice (Biron et al., 1999; Scalzo et al., 1992). We tested whether NK cell activity

induced by MCMV infection would also require the presence of commensal bacteria. SPF and antibiotics-treated mice were infected with 10^4 or 10^5 plaque-forming units (pfu) of MCMV (Smith strain). Following infection, NK cell activity in antibiotics-treated mice was substantially reduced (Figures 2C,D and S2B). While no virus could be detected in the spleen of SPF mice on day 3 after MCMV infection, substantial viral titers were detectable in germ-free mice, emphasizing their inability to control the virus (Figure 2E). NK cells recognize MCMV-infected cells through the activating receptor Ly49H (Brown et al., 2001), which interacts with the MCMV-encoded protein m157 on the surface of infected cells (Arase et al., 2002). However, Ly49H expression by NK cells from germ-free mice was normal, ruling out a decreased number of Ly49H-expressing NK cells as the basis for their susceptibility to MCMV infection (Figure 1F). The failure to prime splenic NK cells in response to microbial ligands of pattern recognition receptors could be reversed when germ-free mice were re-colonized with a complex normal microbiota (Figures 2F and 2G) or a model flora consisting of eight defined bacterial species (i.e., altered Schaedler's flora) (Figure 2H). Reversal occurred as early as 7 days after re-colonization. Our data from re-colonization and from antibiotic treatment experiments indicate that the impact of commensal microbiota on NK cell function is dynamic and tunable.

We tested whether specific pattern recognition receptors are involved in sensing the commensal microbiota for calibration of NK cell responses in non-mucosal tissues. We investigated NK cell function in mice genetically deficient in pattern recognition receptors or their adaptor proteins MyD88 and TRIF. The mice were primed with microbial ligands or CD40 antibodies, signaling of which is independent of the affected microbe-sensing pathways. Failure of NK cell activation would then indicate a role of that pattern recognition pathway in sensing commensal microbiota and calibrating NK cell function in non-mucosal organs. In mice genetically lacking single TLR or combinations of up to five TLR (namely

TLR2, 3, 4, 7, 9) (Conrad et al., 2009), we found mild but consistently reduced NK cell cytotoxicity (Figures S2C and S2E), whereas IFN- γ production was not impaired (Figures S2D and S2F). In contrast, deficiency of *Myd88*, *Trif*, *Nod1* or *Nod2* had no effect on the function of splenic NK cells (Figures 2I,J and S2G-J). Interestingly, a combined MyD88 and TRIF deficiency (*Myd88^{-/-}Trif^{-/-}* mice) resulted in robust reduction of both NK cell-mediated cytotoxicity (Figure 2I) and IFN- γ production (Figure 2J). Collectively, these data indicate that both MyD88 and TRIF-dependent microbial sensors contribute to the microbiota-dependent calibration of NK cell responsiveness.

Defective NK cell priming in germ-free mice

Responsiveness of NK cells is controlled at two central checkpoints. In a developmental process referred to as “education” or “licensing”, NK cells must acquire a balanced input of activating and inhibitory signals, which are regulated by the expression levels of the corresponding activating/inhibitory receptors. Defects in NK cell education are observed in mice lacking ligands for inhibitory NK cell receptors (Fernandez et al., 2005; Kim et al., 2005). However, the acquisition of self class I MHC-specific inhibitory receptors (i.e., Ly49C/I and NKG2A in B6 mice) (Figure 1F), as well as the expression of class I MHC molecules (i.e., H2-D^b and H2-K^b in B6 mice) was normal in germ-free B6 mice (Figure S3A). Although no cell surface marker is available to faithfully tag “educated” NK cells, KLRG1⁺ cells were previously found to be enriched within this population (Fernandez et al., 2005). KLRG1 expression did not differ between NK cells from conventional and germ-free mice (Figure 1C). Once NK cells have been “educated”, they can be rendered fully functional in a second process referred to as “priming,” which is mediated by mononuclear phagocytes such as DC (Kang et al., 2008; Lucas et al., 2007; Mortier et al., 2008). To test whether NK cell unresponsiveness in germ-free mice is NK cell-intrinsic or instead reflects defects in NK

cell priming by DC, we transferred highly purified NK cells from germ-free (CD45.2) into SPF mice (CD45.1) and measured the activity of host and donor-derived NK cells following application of poly(I:C) (Figure 3A). Although NK cells could not be primed following injection of microbial ligands into germ-free mice (Figures 1G and 1H), NK cells from germ-free mice reacted comparable to host-derived cells when transferred into SPF mice (Figure 3B). Thus, consistent with their normal differentiation and maturation (Figures 1A-F), NK cells developing in germ-free mice can, in principle, react to priming signals. These data suggest that defective NK cell function in germ-free mice is NK cell-extrinsic and may reflect defects in the process of “priming”. We tested this hypothesis by transferring NK cells from SPF mice (CD45.1) into control mice or germ-free mice (CD45.2) (Figure 3C). While NK cells from SPF mice transferred into SPF mice were normally primed (Figure 3D), splenic NK cells from SPF mice could not be primed once transferred into germ-free mice (Figure 3E). Thus, the defect in priming in germ-free mice is NK cell-extrinsic and reflects deficient priming signals by bystander cells, most likely DC or other mononuclear phagocytes.

Defective IFN-I response of mononuclear phagocytes in germ-free mice

We, and others, have previously shown that NK cell priming is a result of DC trans-presenting IL-15 after receiving an IFN-I stimulus (Lucas et al., 2007; Mortier et al., 2008; McCartney et al., 2009). Consistent with a previous report (Wilson et al., 2008), absolute and relative numbers of splenic mononuclear phagocyte subsets, including CD8 α ⁻ and CD8 α ⁺ DC and macrophages (i.e., CD11b⁺F4/80⁺ cells), were comparable between SPF and germ-free mice (Figures 4A-D). However, after stimulation with microbial ligands, DC of germ-free mice did not show increased expression of *Il15* (Figure 4E) or IL-15R α (Figure 4F). Thus, DC and macrophages are normally represented in the absence of commensal bacteria in non-mucosal organs but may not provide NK cell priming signals.

Upregulation of IL-15R α in response to microbial stimuli is IFN-I-dependent and does not occur in *Ifnar*-deficient mice (Lucas et al., 2007; Mattei et al., 2001; McCartney et al., 2009). As IFNAR expression by DC of germ-free mice was normal (Figure S3B), we reasoned that the diminished expression of IL-15 and IL-15R may reflect reduced IFN-I production in response to microbial stimulation. High levels of IFN- α and IFN- β were detectable in the serum of SPF mice but not in the serum of germ-free mice after stimulation with poly(I:C) (Figure 4G), LPS (Figure 4H) or after infection with MCMV (Figure 4I). Similar results were obtained from mice in which the commensal microbiota was eradicated with antibiotics (Figures S3C and S3D). Receipt of IFN-I signals induces a transcriptional program required to limit viral infections (Kawai and Akira, 2006). In germ-free mice, IFN-I response genes such as *Irf7* and *Cxcl10* were not up-regulated following microbial stimulation (Figure S3E).

To better visualize IFN-I production in the various organs of mice, we used mice carrying a luciferase reporter gene inserted into the transcriptional start of the *Ifnb1* gene (*Ifnb1*^{+Luc}) to allow faithful tracking of IFN- β expression (Lienenklaus et al., 2009). After microbial stimulation, luciferin was injected and IFN- β production visualized by bioluminescence. Consistent with our serum IFN-I measurements, injection of poly(I:C) into *Ifnb1*^{+Luc} mice housed under SPF conditions led to luciferase expression after 90 min including spleen, lungs and gastrointestinal tract (Figures S4A and S4B). Interestingly, antibiotic-treated *Ifnb1*^{+Luc} mice showed reduced luciferase expression in all organs tested, both mucosal and non-mucosal (Figures 5A and S4B). In order to identify the cellular sources of IFN-I in the spleen, where NK cells are primed, we used mice allowing for Cre-dependent replacement of the *Ifnb1* coding sequence by a luciferase reporter which is then driven by the endogenous *Ifnb1* promoter (Solodova et al., 2011). We analyzed mice in which

Ifnb1 expression is visible in CD11c⁺ DC (*Ifnb1*^{+/*CD11c-luc*} mice) or in all macrophages and neutrophils (*Ifnb1*^{+/*LysM-luc*} mice). Injection of poly(I:C) into *Ifnb1*^{+/*Luc*}, *Ifnb1*^{+/*CD11c-luc*} or *Ifnb1*^{+/*LysM-luc*} mice demonstrated that splenic CD11c⁺ DC are a substantial source of IFN-β (ca. 70% of overall IFN-β production) whereas macrophages or neutrophils contributed less (< 30%) (Figure 5B), which is consistent with previous data (Longhi et al., 2009; McCartney et al., 2009; Scheu et al., 2008).

Our data demonstrate that mononuclear phagocytes from germ-free mice cannot produce IFN-I following microbial stimulation. This can be explained either by defects intrinsic to mononuclear phagocytes or by an environment that suppresses IFN-I responses. To discriminate between these two models, we purified CD11c⁺ DC from SPF and germ-free mice and stimulated them *in vitro* to diminish effects extrinsic to DC. Intriguingly, highly purified DC from germ-free mice did not produce IFN-I in response to microbial stimulation whereas DC from SPF mice produced high levels of IFN-I (Figure 5C). The failure of mononuclear phagocytes from germ-free mice to produce IFN-I is apparently cell intrinsic (Figure 5C) and regulated on the level of transcription because only low levels of *Ifna4* and *Ifnb1* transcripts were found in splenocytes or purified CD11c⁺ splenic DC following injection of microbial ligands (Figure 5D).

Finally, we tested whether exogenously supplied IFN-I could rescue NK cell responsiveness in germ-free mice. Consistent with the normal expression of IFNAR in germ-free mice (Figure S3B), IFN-I injections normalized NK cell responses (Figures 5E and 5F) and led to normal induction of IFN-I target genes such as *Irf7* and *Cxcl10* (Figure S4C). In addition, injection of IFN-I during MCMV infection restored virus control in germ-free mice (Figure 5G). Collectively, the data demonstrates that defective NK cell responses in germ-free

mice are due to a systemic failure of mononuclear phagocytes such as DC to produce IFN-I in response to microbial stimulation.

Our data indicates that signals from the commensal microbiota condition mononuclear phagocytes for efficient IFN-I production. We questioned whether any other form of continuous immune activation could act to condition mononuclear phagocytes for IFN-I production. We observed that germ-free mice were unable to clear lymphocytic choriomeningitis virus (LCMV) infection at day 4 (Figure S4D) and day 8 following infection (Figure S4E). We therefore tested whether continuous immune activation through a persisting virus could normalize IFN-I production. While germ-free mice were unable to produce IFN-I in response to poly(I:C) injections prior to virus infection (Figure S4F), IFN-I production was comparable to conventional mice after 8 days of continuous infection (Figure S4G).

The commensal microbiota controls the setpoint for cytokine production by mononuclear phagocytes

To gain further insights into the gene expression programs of mononuclear phagocytes, we analyzed expression of additional microbe-inducible genes. In contrast to IFN-I gene expression, LPS-induced up-regulation of CD86 (Figure 6A), *Ccl5* (chemokine (C-C motif ligand 5) and *Fpr1* (formyl peptide receptor 1) was normal or even increased in germ-free mice (Figure 6B), indicating that they are not globally unresponsive to microbial stimulation. However, in addition to IFN-I genes, inflammatory response genes encoding cytokines such as *Il6*, *Tnf*, *Il12* and *Il18* were not expressed following microbial stimulation of DC from germ-free mice (Figures 6C-F). Similar to IFN-I, this block is transcriptional as less cytokine mRNA could be detected (Figure 6G). The failure of germ-free mice to produce IL-6 or TNF does not reflect a lack of sensitivity in mononuclear phagocytes to IFN-I or IFN- γ because

Ifnar^{-/-} and *Ifng*^{-/-} mice showed virtually normal serum levels of these cytokines following microbial stimulation (Figure S5). Thus, cues from the commensal microbiota are not required for the expression of all genes induced by microbial stimulation but rather for a certain set of genes, including IFN-I and other inflammatory cytokines. This indicates that signals from the commensal microbiota functionally adapt innate immune system components to a state in which they can respond to microbial stimulation with the production of cytokines required for immunity to viruses and bacteria.

Why are microbial stimuli not appropriately translated into the expression of pro-inflammatory genes? Expression of the relevant microbe-sensing molecules such as TLR or cytoplasmic nucleotide sensors (i.e., Mda5) and the cytoplasmic signaling adaptor molecules required for IFN-I gene expression were normal, or even increased, in DC and splenocytes of germ-free mice (Figures S6A and S6B). We considered whether the transduction of signals downstream of these receptors is defective in germ-free animals. The various pattern recognition receptor signaling pathways converge on IRF3 and NF- κ B, which, upon activation, translocate to the nucleus and induce transcription (Kawai and Akira, 2006). Both IRF3 and NF- κ B are required for optimal induction of IFN-I expression (Smale, 2010; Tamura et al., 2008). Interestingly, the amount of phosphorylated I κ B α (Figures 7A and S6C) and of IRF3 (Figures 7B and S6D), as well as their nuclear translocation (Figure 7C), was not impaired in mononuclear phagocytes isolated from poly(I:C) injected germ-free mice. These data demonstrate that the central microbe-induced signaling pathways and nuclear translocation of major signaling intermediates required for the transcription of inflammatory genes are not defective in germ-free mice.

Normal signaling activity was contrasted by our discovery that transcription of certain inflammatory genes was stalled in the absence of commensal microbiota (Figures 5D and

6G), while others occurred normally (Figure 6B). These findings may be explained by chromatin level changes controlled by cues from commensal microbiota. In the absence of these cues, activated transcription factors may not bind to the promoter and enhancer elements of their respective cytokine loci or may fail to recruit RNA Pol II. We performed chromatin immunoprecipitation (ChIP) assays, in which we tested whether NF- κ B p65, IRF3 and RNA Pol II can bind to the promoters of genes, transcription of which is dependent (e.g., *Ifnb1*, *Il6*, *Tnf*) or independent (e.g., *Cd86*) of the commensal microbiota. Stimulation of DC from SPF mice led to rapid recruitment of NF- κ B p65, IRF3 and RNA Pol II to the promoter regions of all genes investigated. However, they could not be recruited to the promoter regions of those genes whose expression cannot be induced in germ-free DC (Figures 7D and 7E). Importantly, chromatin binding of NF- κ B p65 and RNA Pol II recruitment to the *Cd86* promoter was normal in germ-free mice (Figures 7D and 7E). These data demonstrate that the failure of mononuclear phagocytes to produce inflammatory cytokines reflects defects at the chromatin level that prevent binding of central transcription factors.

We have begun to address whether this is due to repressive or permissive chromatin marks involved in the expressional regulation of certain cytokine genes. Interestingly, and consistent with previous data (Ramirez-Carrozzi et al., 2009), analysis of DC from conventional mice for trimethylation of H3K4, a histone mark strictly correlated with transcriptionally active or poised genes, revealed that H3K4me3 was present at or around the transcriptional start sites of most inflammatory response genes (Figure 7F). Highest levels of H3K4me3 were found at primary response genes containing CpG island promoters (e.g., *Tnf*) and lower but detectable levels were found at the transcriptional start sites or promoters of secondary response genes with non-CpG island promoters (e.g., *Il6*) as reported previously (Ramirez-Carrozzi et al., 2009). Importantly, this activating histone mark was reduced at the promoters of microbiota-dependent genes in germ-free mice (Figure 7F) and erased in DC of

mice treated with antibiotics (Figure S6E). Collectively, the data indicate that signals from the commensal microbiota may introduce tunable chromatin level changes such as the deposition of activating histone marks poisoning the expression of pro-inflammatory cytokine genes.

Histone marks are under the complex control of enzymes catalyzing the addition or removal of methyl and acetyl groups from histone tails. Acetylation of histones has, without exception, been correlated with transcriptional activation. We explored the role of histone acetylation for IFN-I production by treating DC from germ-free mice with inhibitors of histone deacetylases (HDACi) to enhance acetylation of histone proteins. If histone acetylation is low in DC of germ-free mice, HDACi treatment may restore responsiveness to microbial stimulation. Interestingly, HDACi treatment of DC isolated from germ-free mice did not restore production of IFN-I, IL-6 or TNF (Figures S6F and S6G). It should be noted, though, that HDACi act globally and, in addition to their effects on histones, affect acetylation of proteins and transcription factors. Thus, data obtained with such compounds are difficult to interpret.

Methylation of DNA is another important mode of epigenetically regulating gene expression. DNA methylation is associated with a repressed chromatin state and inhibition of gene expression (Klose and Bird, 2006). It has been documented that the CpG island promoter of the *Tnf* gene is densely methylated in non-hematopoietic cells repressing *Tnf* expression (Kruys et al., 1993). We tested whether removal of methyl groups from chromatin by treating DC from germ-free mice with 5-azacytidine would restore responsiveness to microbial stimulation. DC isolated from 5-azacytidine-treated SPF mice produced more IFN- β (Figures 7G and 7H), IL-6 and TNF compared to controls (Figure S6H). Interestingly, demethylating agents did not confer responsiveness to DC from germ-free mice indicating that differences in

DNA methylation are unlikely to fully explain why DC from germ-free mice do not react to microbial stimulation.

Discussion

The innate immune system has been widely perceived as a system ready for immediate reaction upon encountering pathogens. Mononuclear phagocytes are phagocytic cells such as macrophages and DC that express pattern recognition receptors, the ligation of which leads to the induction of an inflammatory gene expression program required for an effective and concerted response against pathogens. Such responses were believed to be innate, cell autonomous and independent of any adaptive traits. Our data now demonstrates that readiness of mononuclear phagocytes to respond to microbial signals with the expression of various cytokine genes requires calibration by signals originating from the commensal microbiota. One important family that required signals from commensals were the various IFN-I genes. Consequently, the IFN-I response in germ-free mice was impaired, leading to reduced priming of cytotoxic lymphocytes such as NK cells and susceptibility to viral infections. A previous report showed that germ-free mice are susceptible to mucosal influenza A virus infection (Ichinohe et al., 2011). Defective inflammasome activation was found to impair migration of mucosal, respiratory tract DC to the mucosa-draining lymph nodes, resulting in reduced priming of anti-viral T cell responses. However NK cell activation and IFN-I production were only mildly affected in mice genetically lacking IL-1 or IL-18, cytokines required for resistance to influenza infection. Thus, the commensal microflora controls distinct programs in mononuclear phagocytes from both mucosal and non-mucosal lymphoid organs.

It was unexpected that the commensal microbiota had such a profound impact on the readiness of mononuclear phagocytes residing in non-mucosal lymphoid organs. There is a paucity of data concerning the cytokine responses of non-mucosal mononuclear phagocytes from germ-free mice. Collective evidence from early analyses reveals that differentiation and maturation of macrophages (Bauer et al., 1963; Morland et al., 1979; Shelton et al., 1970) and DC (Walton et al., 2006; Wilson et al., 2008) is not dependent on commensals. However, antimicrobial function may be impaired as a result of reduced production of cytokines known to enhance antimicrobial activity (Mitsuyama et al., 1986; Morland et al., 1979). A recent report has provided a possible framework for how tissue-resident immune cells in non-mucosal organs receive signals from commensal bacteria (Clarke et al., 2010). Antimicrobial function of neutrophils requires NOD1-dependent sensing of peptidoglycan by bone marrow neutrophil precursors. Although living bacteria likely do not pass the mesenteric lymph node filter (Macpherson and Uhr, 2004), gut microbiota-derived peptidoglycan could be detected in the serum of mice, indicating that microbial ligands of pattern recognition receptors circulate through the body at sufficient concentrations to influence function of immune cells at non-mucosal sites (Clarke et al., 2010). While this study provides a rationale for how microbiota signals may be communicated to sterile peripheral lymphoid organs, NOD1-dependent sensing of commensal microbiota is likely not required for poising of IFN-I expression by mononuclear phagocytes because NK cell activation was virtually normal in *Nod1*^{-/-} mice. Therefore, future studies will need to address the molecular cues from commensal microflora and their sensors required to poise IFN-I expression in non-mucosal lymphoid organs.

The experiments using antibiotics-treated and germ-free mice re-colonized with bacteria show that the changes induced by commensal microbiota are reversible and possibly tunable. The loss of IFN-I production and resistance to viral infection after treatment of SPF mice with antibiotics may reflect direct functional tuning of tissue-resident splenic DC or their

circulating progenitors. Previous parabiosis experiments indicated that splenic DC were entirely replaced by circulating progenitors within 10-14 days (Liu et al., 2007) and the loss of NK cell activity in our study required treatment with antibiotics for at least 7 days. It is unlikely that production of suppressive factors extrinsic to mononuclear phagocytes were the basis for their failure to respond to microbial stimulation as highly purified mononuclear phagocytes remained unresponsive when stimulated *in vitro*. Collectively, our data provides a novel perspective as to how changes in intestinal microflora introduced by antibiotic therapies, hygiene or lifestyle factors may tune the effector program of mononuclear phagocytes in non-mucosal organs.

The exact mechanisms underlying microbiota-induced poising of gene expression in mononuclear phagocytes need further analyses. Our data demonstrated that the major pattern recognition receptor signaling pathways (NF- κ B and IRF3 activation) and their nuclear translocation were not impaired in germ-free animals. In addition, not all myeloid genes induced by microbial stimulation were defective, indicating that specific groups of genes required poising by signals from commensal microbiota whereas others did not. Interestingly, both primary and secondary inflammatory response genes (as defined by Smale, 2010) showed a dependence on signals from commensal microflora. These data make it unlikely that the underlying mechanism for failure to express certain genes in germ-free mice is based upon previously recognized differences in the transcriptional activation of these classes of genes (Hargreaves et al., 2009; Smale, 2010). The signaling and transcription factor requirements for expression of affected versus unaffected genes were not obviously different, suggesting chromatin level, epigenetic changes as the most likely explanation. Our data demonstrated that NF- κ B p65, IRF3 and RNA Pol II cannot be recruited to the promoter regions of genes affected by the absence of commensal microbiota, whereas inflammation-induced binding to loci unaffected by the presence of microbiota was normal. These data support the concept that

chromatin binding of crucial transcription factors, and initiation of transcription, is defective in the microbiota-dependent genes. Gene-specific regulation occurs at the level of chromatin and includes DNA methylation, covalent histone modifications and nucleosome remodeling (Smale, 2010; Klose and Bird, 2006). We have initiated studies into permissive and repressive chromatin marks at the promoter regions of microbiota-dependent genes. H3K4me3, a chromatin mark characteristic of active and poised genes (Santos-Rosa et al., 2002; Wei et al., 2009), was constitutively present at the promoter regions of inflammatory response genes in primary splenic DC, as previously indicated (Hargreaves et al., 2009; Ramirez-Carrozzi et al., 2009). Interestingly, in germ-free mice, trimethylated H3K4 was low at microbiota-dependent genes of both classes but normally represented at other inflammatory response genes independent of poisoning by microbiota. Treatment of conventional mice with antibiotics erased H3K4me3 from inflammatory response genes, indicating that commensal microbiota may introduce tunable chromatin level changes such as the deposition of activating histone marks. Gene-specific regulation by tuning of chromatin was also shown to underlie the phenomenon of LPS tolerance (Chen and Ivashkiv, 2010; Foster et al., 2007). Although 5-azacytidine treatment of DC from SPF mice led to enhanced production of cytokines, it did not restore cytokine production in germ-free DC. These data indicate that DNA hypermethylation is unlikely to be the underlying epigenetic signature repressing cytokine production by DC from germ-free mice. Future studies from genome-wide assessments of DNA methylation, various activating and repressive histone marks, as well as of their regulators, will reveal a more complete picture of the global chromatin states instructed by signals from commensal microbiota.

Poising of gene expression within mononuclear phagocytes is likely to commence with colonization of newborns by increasingly complex microbiota. It is intriguing to speculate that colonization of mucosal surfaces controls the development of a myeloid functional

program designed to manage postnatal exposure to microbes and pathogens. During fetal development, the main function of macrophages is the removal of apoptotic cells generated in developmental processes (Ovchinnikov, 2008). Expression of inflammatory genes by fetal mononuclear phagocytes may not be desirable. However, after birth, when mucosal surfaces become entry ports for pathogens, inflammatory genes are necessary to coordinate powerful and effective antimicrobial immunity. The commensal microbiota induces genome-wide changes, likely at the level of chromatin. This leads to poising of inflammatory gene expression, thereby calibrating mononuclear phagocytes at sterile, non-mucosal sites to respond promptly to pathogens.

Experimental Procedures

Mice

Conventional C57BL/6 mice were purchased from Janvier. Germ-free mice were generated as described before and bred in our facilities (Sanos et al., 2009) or were purchased from the University Hospital Bern. Information about additional strains can be found in the Supplemental Experimental Procedures.

Antibiotic treatment

For eradication of the commensal microbiota, drinking water was provided containing Cefoxitin (Santa Cruz Biotechnology), Gentamicin (Sigma), Metronidazol (Sigma) and Vancomycin (Hexal).

Virus infections

For MCMV infections, mice were injected i.v. with 10^4 to 10^6 pfu MCMV (Smith strain). Viral titers in spleen were determined in a standard MCMV plaque assay (see Supplemental

Experimental Procedures). For LCMV infections, groups of mice were injected i.v. with 200 pfu LCMV (WE strain). Viral load in different organs was determined by plaque assay from organ samples on day 4 and day 8 after infection (see Supplemental Experimental Procedures).

***Ex vivo* analysis of NK cell function**

NK cell cytotoxicity was determined in a standard 4 h ⁵¹Cr release assay against YAC-1 target cells (Lucas et al., 2007). Cytokine production by NK cells was determined by intracellular cytokine staining as described previously (Lucas et al., 2007).

Adoptive transfer experiments

Splenic NK cells from conventional or germ-free mice were highly purified as described before (Lucas et al., 2007). 1.5×10^6 sorted NK cells were transferred intravenously into germ-free or SPF mice.

Determination of serum cytokine levels

IFN-I concentrations were measured by IFN- α or IFN- β ELISA (PBL Interferonsource) or by VSV bioassay (see Supplemental Experimental Procedures). TNF, IL-6, IL-12 (all from R&D Systems) and IL-18 (MBL) were detected by sandwich ELISA.

Cell isolation and flow cytometry

Splenocytes were isolated, stained for cell surface and intracellular markers and analyzed as previously described (Vonarbourg et al., 2010). A complete list of antibodies can be found in the Supplemental Experimental Procedures.

Bioluminescence measurements

Mice were injected i.p. with luciferin (Caliper) at a final concentration of 150 mg/kg body weight. Within 30 min., whole body imaging was performed or mice were sacrificed and organs were individually analyzed using a Caliper Life Science IVIS Spectrum camera. For *ex vivo* analysis, the indicated organs were removed for homogenization and lysed in Cell Culture Lysis Buffer (Promega) using Lysing Matrix A on a FastPrep-24 (MP Biomedicals). Luciferase activity was measured in a Sirius Tube Luminometer (Berthold Technologies) using the single Luciferase Assay System (Promega).

Chromatin immunoprecipitation (ChIP)

Splenic DC were isolated using CD11c beads (Miltenyi). DC (3×10^6) were stimulated *in vitro* with 0.1 $\mu\text{g/ml}$ LPS for the indicated time. Cells were washed with PBS and cross-linked with 1% formaldehyde for 8 min at room temperature. ChIP for RNA Polymerase II was performed as previously described (Kiss et al., 2011). Minor changes are described in the Supplemental Experimental Procedures. ChIP for transcription factors and histone modifications was performed as previously described (Saccani et al., 2002).

Statistical analysis

Student's t test was used to determine the significance of the data sets. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. = not significant.

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Supplemental information

Supplemental information includes Figures S1-S6, Supplemental Experimental Procedures and Supplemental References.

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Figure legends

Figure 1: Impaired NK cell responses following microbial stimulation in germ-free mice

(A,B) Splenocytes from specific pathogen-free (SPF) or germ-free (GF) mice were stained with antibodies specific for CD3 and NK1.1 and analyzed by flow cytometry. Percentages (\pm SD, $n \geq 5$) and absolute numbers (\pm SD, $n \geq 5$) of NK1.1⁺ CD3⁻ NK cells were determined.

(C) Splenocytes from SPF and GF mice were stained with antibodies specific for CD3, NK1.1 and KLRG1 and analyzed by flow cytometry. Histograms show staining with KLRG1 antibody (grey) or isotype control antibody (open) of NK1.1⁺ CD3⁻ cells. Numbers represent percent KLRG1⁺ cells.

(D) Splenocytes from SPF and GF mice were stained with antibodies specific for CD3, NK1.1, CD11b and CD27 and analyzed by flow cytometry. Dot plots are gated on NK1.1⁺CD3⁻ cells, numbers represent percent cells in quadrant. Bar diagram shows the mean percentage (\pm SD, $n = 7$) of NK cells in each subset.

(E,F) Splenocytes from SPF and GF mice were stained with antibodies specific for CD3, NK1.1 and the indicated surface markers and analyzed by flow cytometry. Histograms are electronically gated on NK1.1⁺CD3⁻ cells and show co-staining with the indicated marker (grey) or isotype control antibody (open). Numbers represent percent cells expressing the indicated cell surface marker.

(G) Groups of SPF and GF mice were injected with poly(I:C). Granzyme B expression by splenic NK cells was determined by intracellular staining and flow cytometry 14 h after poly(I:C) injection. Dot plots are gated on CD3⁻NK1.1⁺ NK cells. Numbers in gates show percent granzyme B-positive NK cells.

(H,I) Groups of SPF or GF mice were injected with the indicated TLR ligands, anti-CD40 or PBS (control). Cytotoxicity (H) and IFN- γ production (I) of NK cells against YAC-1 targets 14-16 h after stimulation. The percentage of NK1.1⁺CD3⁻ cells in the lymphocyte populations was determined prior to the cytotoxicity assay and lymphocyte numbers were adjusted to

contain the same number of NK cells. Data represent mean percentage specific lysis (error bars were omitted for clarity) or mean (\pm SD, $n \geq 3$) percentage of NK cells producing IFN- γ . Data are representative of two (G) or at least three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, n.s. = not significant. See also Figure S1.

Figure 2: NK cell responsiveness is dynamically regulated by the commensal microbiota

(A,B) SPF mice were given antibiotics in the drinking water for 10 days and then injected with poly(I:C). Cytotoxicity (A) and IFN- γ production (B) of splenic NK cells against YAC-1 targets 14-16 h after stimulation. Data represent mean (\pm SD, $n = 3$).

(C,D) Groups of antibiotic-treated SPF mice and control mice were infected with 1×10^4 pfu MCMV. Cytotoxicity (C) and IFN- γ production (D) of splenic NK cells against YAC-1 targets 24 h after stimulation. Data represent mean (\pm SD, $n = 3$).

(E) SPF and GF mice were infected with 1×10^6 pfu MCMV. Viral titers in the spleen were determined on day 3.

(F,G) Groups of GF mice were re-colonized with a complex microbiota and injected with PBS or poly(I:C) 10 days later. Cytotoxicity (F) and IFN- γ production (G) of splenic NK cells from the indicated mouse groups against YAC-1 targets 16 h after stimulation. Data represent mean (\pm SD, $n = 3$).

(H) Groups of SPF, GF and GF NMRI mice re-colonized with Altered Schaedler's Flora were injected with PBS or poly(I:C) and cytotoxicity of splenic NK cells was analyzed against YAC-1 target cells 16 h later. Data represent mean specific lysis ($n = 5$).

(I, J) Cytotoxicity (I) and IFN- γ production (J) of splenic NK cells from the indicated mouse strains against YAC-1 targets 14-16 h after stimulation with CD40 antibody. Data represent mean (\pm SD, $n = 3$).

Data are representative of two (C-E,H-J) or three (A,B,F,G) independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, n.s. = not significant. See also Figure S2.

Figure 3: Failure of NK cell priming in germ-free mice

(A,B) Experimental Design (A). Sorted splenic NK cells from GF mice (CD45.2) were transferred into congenic SPF (CD45.1) mice. Mice were injected with poly(I:C) one day later. IFN- γ production (B) by donor (CD45.2) or host-derived (CD45.1) splenic NK cells against YAC-1 targets was determined after 16 h. Data represent mean (\pm SD, $n = 3$).

(C,D) Experimental Design (C). Sorted splenic NK cells from SPF mice (CD45.1) were transferred into congenic SPF or GF (CD45.2) mice. IFN- γ production (D) by donor (CD45.1) or host-derived (CD45.2) splenic NK cells against YAC-1 targets was determined after 16 h. Data represent mean (\pm SD, $n = 3$). Data are representative of three independent experiments.

Figure 4: Mononuclear phagocytes of germ-free mice do not produce IFN-I

(A,B) Splenocytes from the indicated mice were stained with antibodies specific for CD3, CD19, CD11c and class II MHC (I-A^b) and analyzed by flow cytometry. Numbers in plots are mean percentages ($n = 5$) of gated CD11c⁺I-A^{b+} cells (A) or of CD11b⁺CD8 α ⁻ or CD11b⁻CD8 α ⁺ DC (B).

(C) Relative and absolute splenic DC numbers (mean \pm SD, $n \geq 5$).

(D) Relative and absolute numbers (mean \pm SD, $n \geq 5$) of splenic CD11b⁺F4/80⁺ macrophages.

(E,F) Groups of mice were injected with poly(I:C), splenic DC were isolated after 12 h and *Ill5* expression analyzed using quantitative (q) RT-PCR (E). IL-15R α expression by unstimulated (open) and poly(I:C)-stimulated (grey) cDC.

(G) Groups of mice were stimulated with the indicated TLR ligands and serum IFN-I levels were determined at the indicated time points using the VSV bioassay. Data represent mean (\pm SD, $n \geq 5$).

(H) Groups of mice were stimulated with poly(I:C) and serum IFN- α and IFN- β concentrations were determined at the indicated time points by ELISA. Data represent mean (\pm SD, $n = 3$).

(I) Groups of mice were infected with 1×10^6 pfu MCMV i.v. Serum IFN-I concentrations were determined after 24, 48 and 72 h using the VSV bioassay. Data represent mean (\pm SD, $n = 3$).

Data are representative of two (E,F,I), three (C,D,H) or more (A,B,G) individual experiments.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. = not significant. See also Figure S3.

Figure 5: Systemic failure of pathogen-induced IFN-I production in germ-free mice

(A) Groups of SPF *Ifnb1*^{+Luc} mice were given antibiotics in the drinking water for 10 days and then injected with poly(I:C). After 120 min, luciferase activity was determined using a bioluminescence camera. Representative pairs of mice from 3 individual experiments are shown.

(B) Groups of *Ifnb1*^{+Luc}, *Ifnb1*^{+CD11c-luc} and *Ifnb1*^{+LysM-luc} mice were injected with poly(I:C) or PBS (control). Two hours later, luciferase activity was determined in spleen lysates using a luminometer. Graphs show mean (\pm SD, $n = 3$) of relative luciferase units (RLU) per mg spleen.

(C) Purified DC from SPF and GF mice were cultured *in vitro* in the presence of poly(I:C) (5 μ g/ml) or LPS (100 ng/ml) for 16 h. IFN-I levels in the tissue culture supernatants were determined by VSV bioassay. Data represent mean (\pm SD, $n = 5$).

(D) Groups of mice were injected with poly(I:C) or PBS. RNA was prepared from splenocytes or purified splenic DC after 2 h. *Ifnb1* and *Ifna4* expression was analyzed by qRT-PCR. Data represent fold induction (mean \pm SD, n = 5) of gene expression after poly(I:C) stimulus.

(E,F) Groups of mice were injected with poly(I:C), human IFN- α A/D or with PBS (control). Cytotoxicity (E) and IFN- γ production (F) of splenic NK cells against YAC-1 targets 16 h post stimulation. Data represent mean (\pm SD, n = 3).

(G) Control and antibiotic-treated SPF mice were infected with 1×10^6 pfu MCMV i.v. Half of the antibiotic treated mice received 0.5×10^4 U murine IFN- β at 24 h intervals, starting at the time point of infection. Viral titers in the spleen were determined on day 3.

Data are representative of two (B,G) or three (A,C-F) individual experiments. ** $p \leq 0.01$, *** $p \leq 0.001$, n.s. = not significant. See also Figure S4.

Figure 6: Selective expression of pathogen-inducible genes in germ-free mice

(A) Groups of mice were injected with LPS (grey) or left unstimulated (open). After 3 h, CD86 expression by CD11c^{high} MHCII^{high} cells was determined using flow cytometry.

(B) Groups of mice were injected with LPS. *Ccl5* and *Fpr1* expression in the spleen was determined by qRT-PCR at the indicated time points.

(C-E) Groups of mice were stimulated with poly(I:C) or LPS. At various time points, serum IL-6 (C), TNF (D) and IL-12p70 (E) concentrations were determined by ELISA. Data represent mean (\pm SD, n = 3).

(F) Groups of mice were stimulated with LPS. At various time points, serum IL-18 concentrations were determined by ELISA. Data represent mean (n = 2).

(G) Groups of mice were injected with poly(I:C) or LPS. RNA was prepared from splenocytes 1 h (*Tnf*) or 2 h (*Il6*) later. *Il6* and *Tnf* expression were analyzed by qRT-PCR. Data represent fold induction (mean \pm SD, n = 5) of gene expression after the TLR stimulus.

Data are representative of one (F), two or more (C,D) independent experiments. See also Figure S5.

Figure 7: Increased barrier for NF- κ B and IRF3 chromatin binding in germ-free DC

(A,B) Groups of mice were injected with poly(I:C) and splenic DC purified at the indicated time points. Western blot analyses of phosphorylated IRF3 and phosphorylated I κ B α (B) in DC lysates. Densitometric quantification of Western blot analyses of phosphorylated IRF3 (A) and I κ B α (B) relative to loading control (Hsp90). Data represent mean (\pm SD, n = 3).

(C) DC were purified from SPF and GF mice and stimulated *in vitro* in the presence of LPS. Cytosolic and nuclear lysates were prepared at the indicated time points and nuclear translocation of NF- κ B p65 determined by Western blot analysis.

(D) DC were purified from SPF and GF mice and stimulated with LPS *in vitro*. IRF3 and NF- κ B p65 chromatin-immunoprecipitation (ChIP) was performed to determine specific pulldown at the transcription factor binding sites in the promoter regions of the indicated genes. Results represent specific pulldown relative to a DNA input sample. Pulldown with a control antibody was significantly lower or absent.

(E) DC were purified from SPF and GF mice and stimulated with LPS *in vitro*. RNA Pol II ChIP was performed to determine specific pulldown of RNA Pol II at the transcriptional start sites of the indicated genes. Results represent specific pulldown relative to a DNA input sample normalized to pulldown with a control antibody.

(F) DC were purified from SPF and GF mice. H3K4me3 ChIP was performed and specific pulldown was determined around the transcriptional start (TS) sites of the indicated genes. Results represent specific pulldown relative to a DNA input sample. Pulldown with a control antibody was significantly lower or absent.

(G,H) Groups of mice were treated *in vivo* with 5-azacytidine (5-Aza) or left untreated. Subsequently, splenic DC were isolated and stimulated *ex vivo* in the presence of 0.1 μ g/ml

LPS for 4 h. *Ifnb1* mRNA levels were determined by qRT-PCR (G). IFN- β concentration in the culture supernatant was determined by ELISA (H).

Data are representative of two (C,G,H), three (D-F) or four (A,B) independent experiments. n.d. = not detected. See also Figure S6.

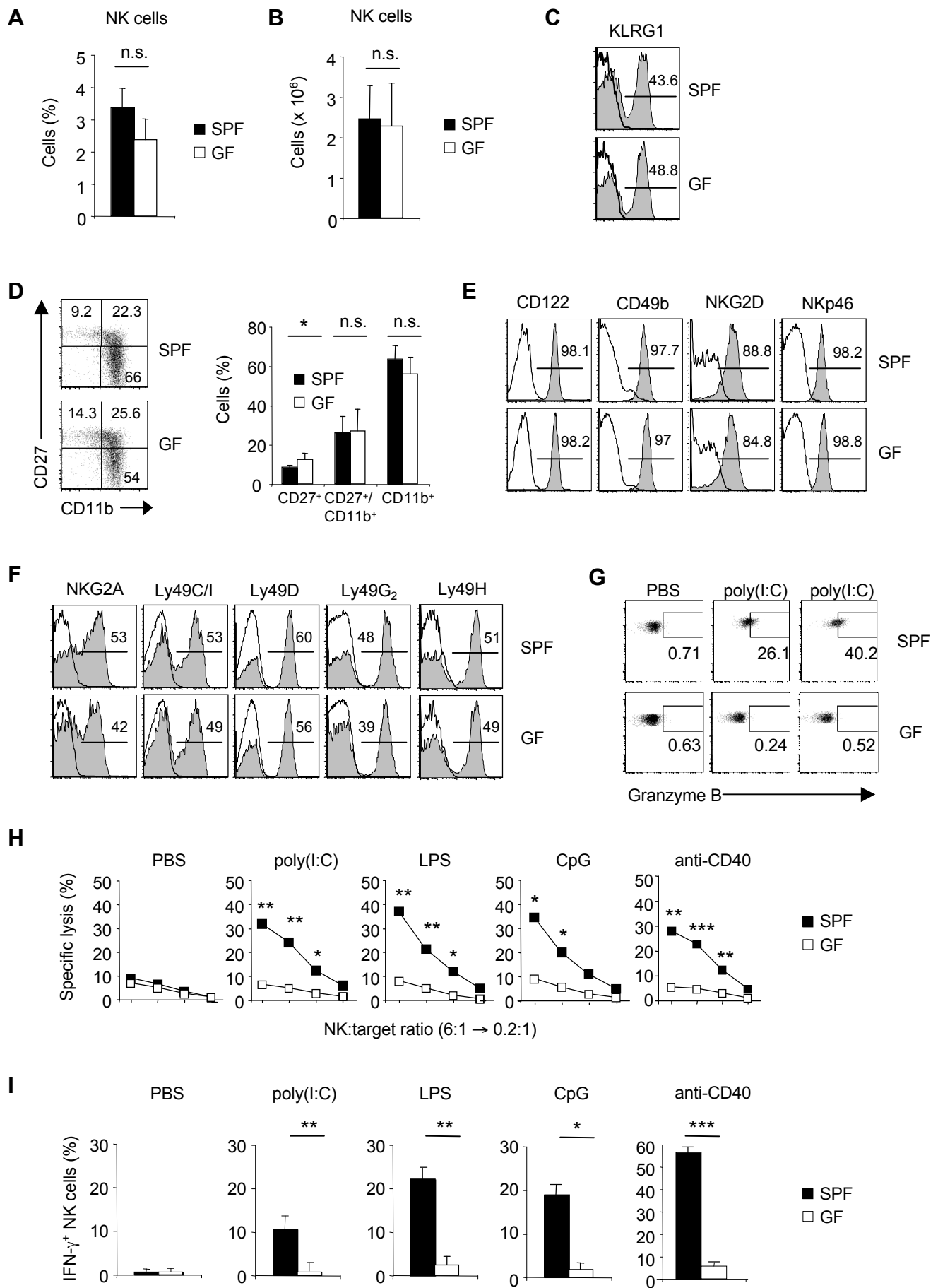


Figure 1 (Ganal et al.)

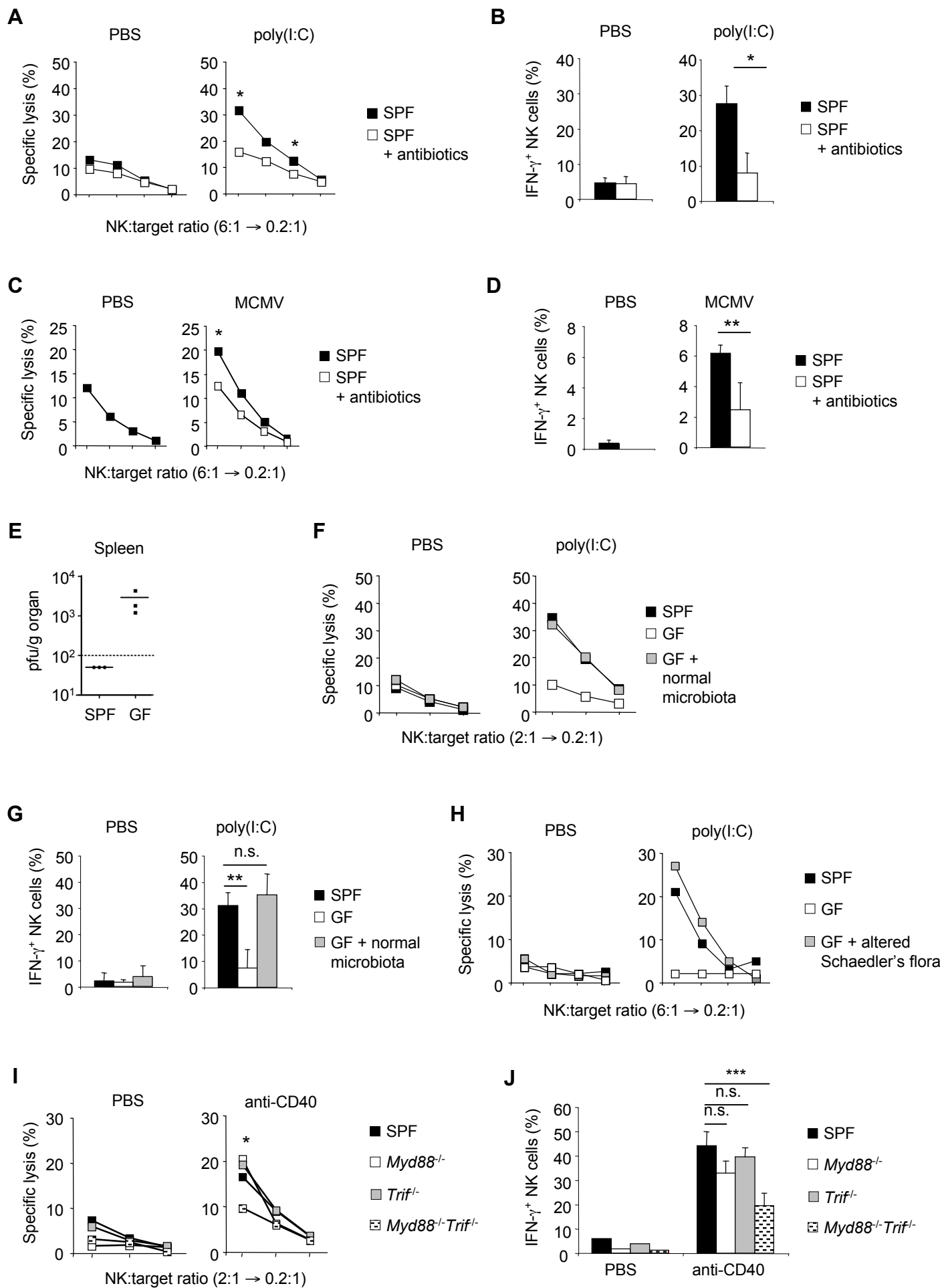


Figure 2 (Ganal et al.)

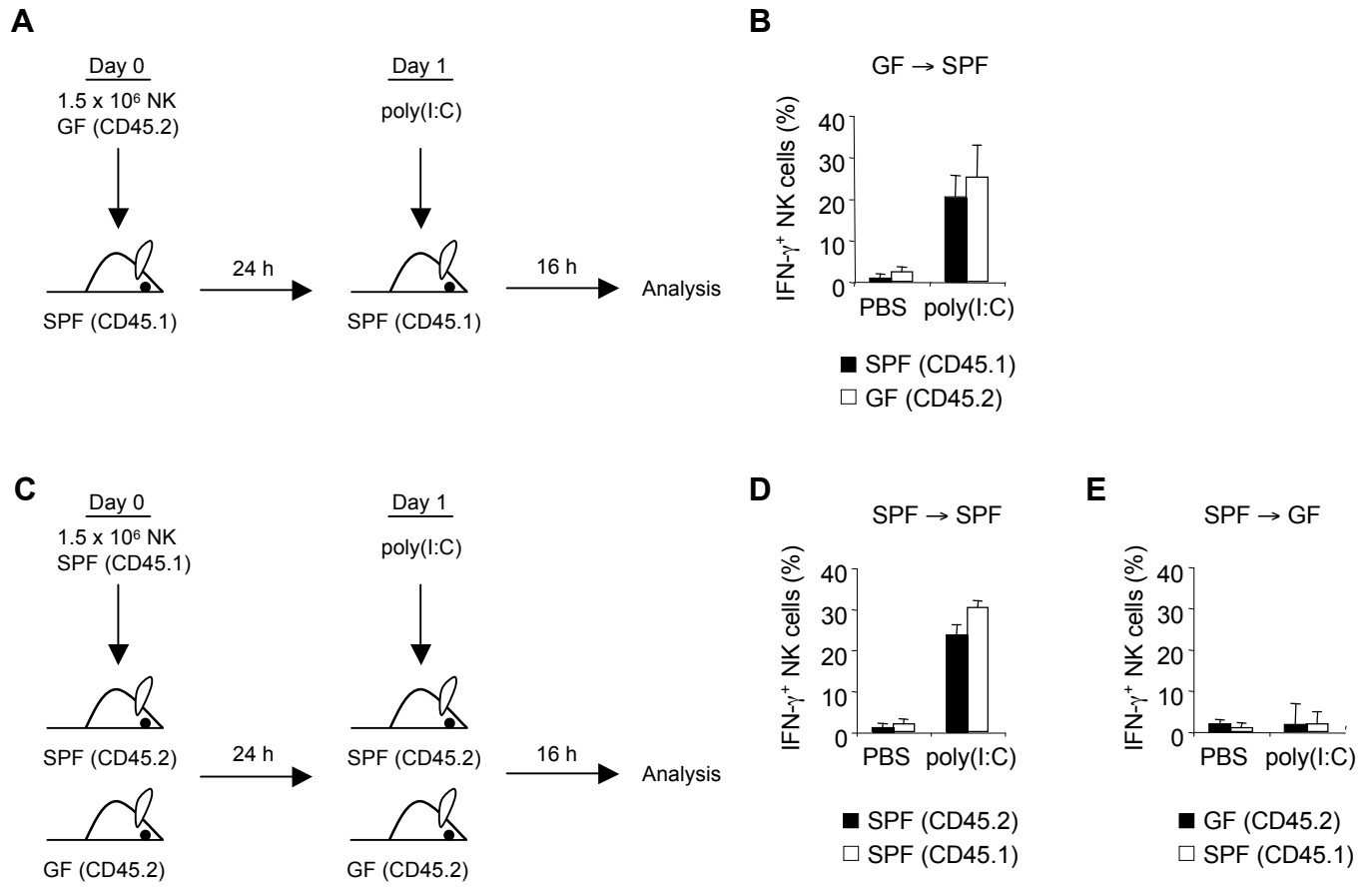


Figure 3 (Ganal et al.)

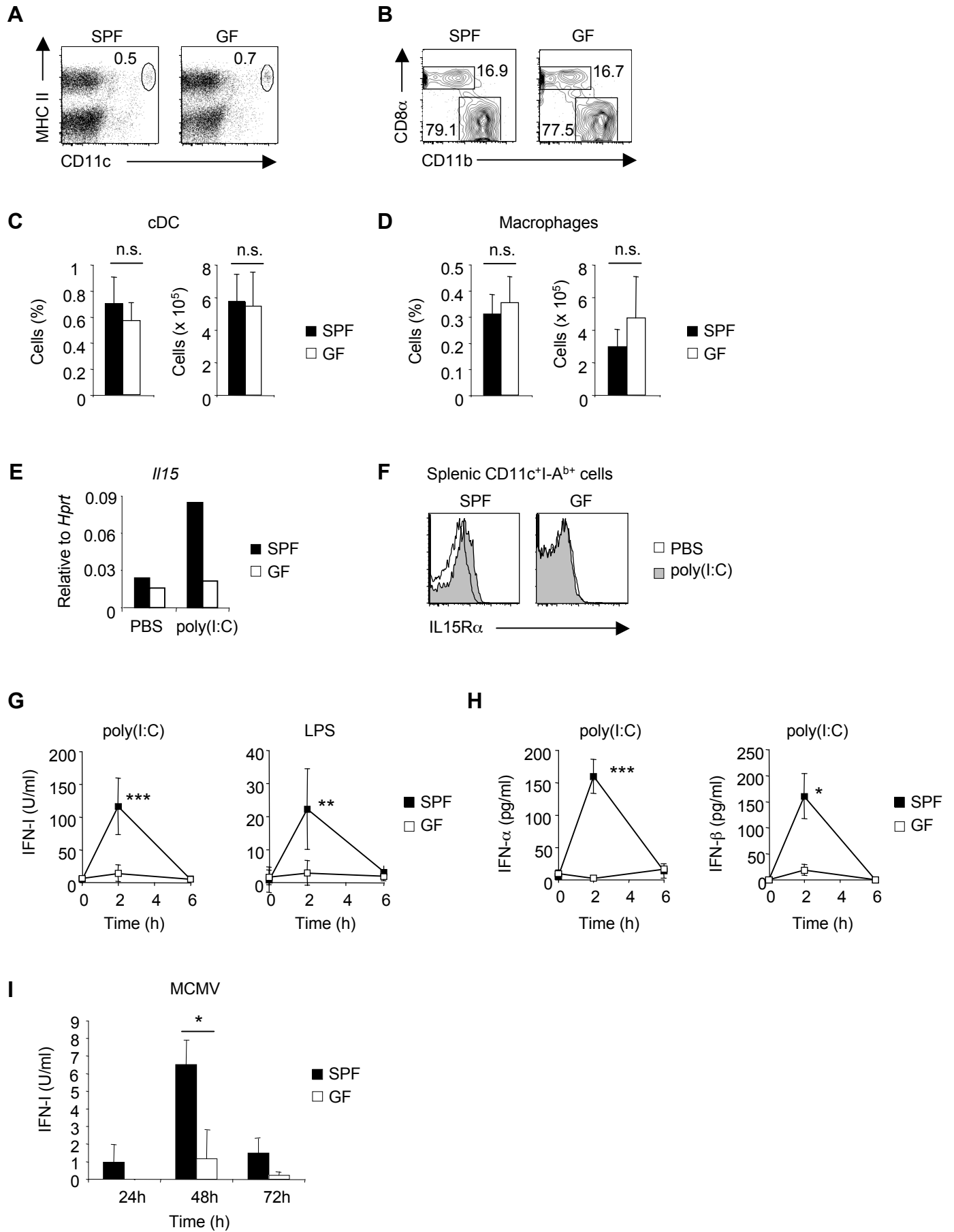


Figure 4 (Ganal et al.)

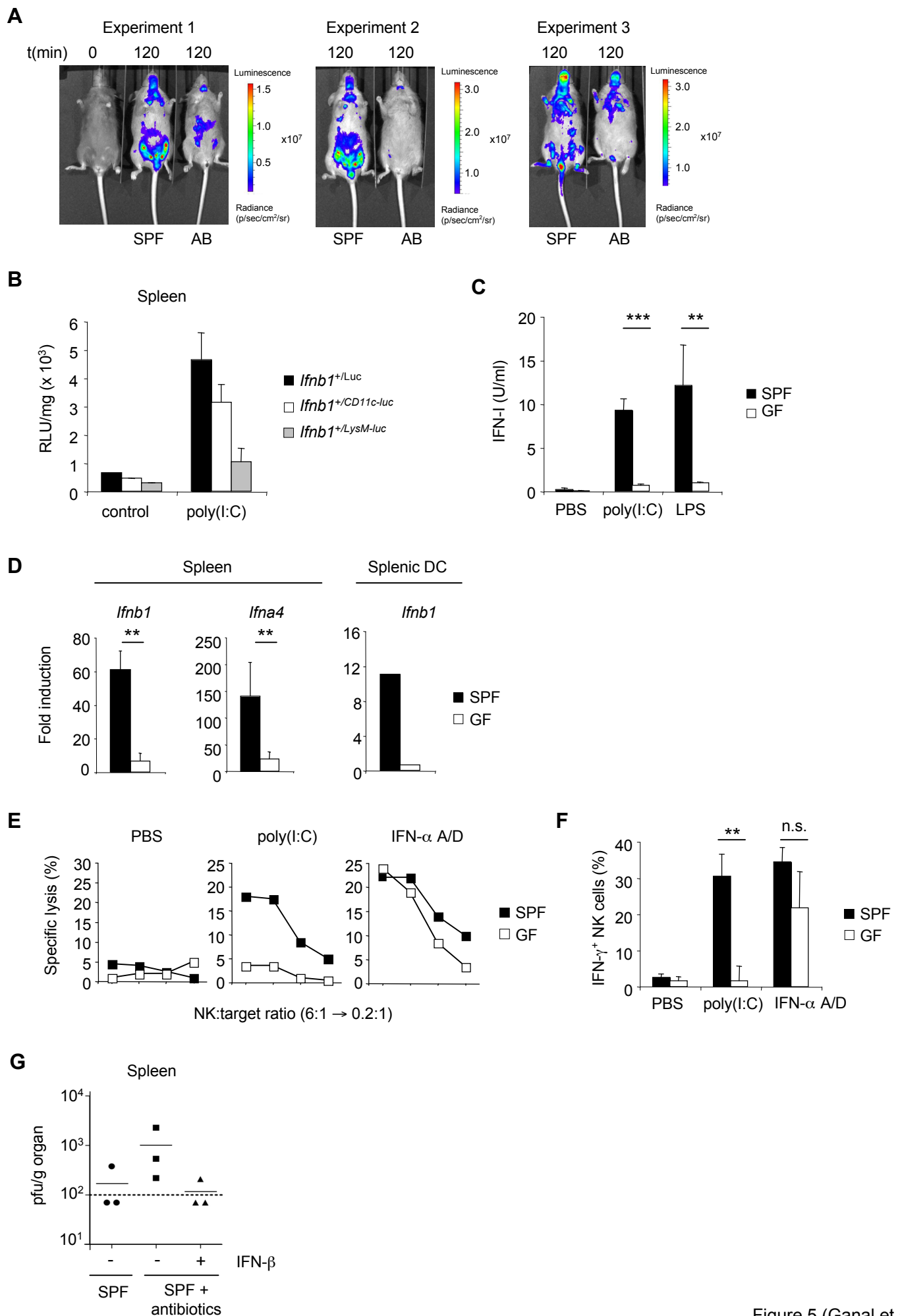


Figure 5 (Ganal et al.)

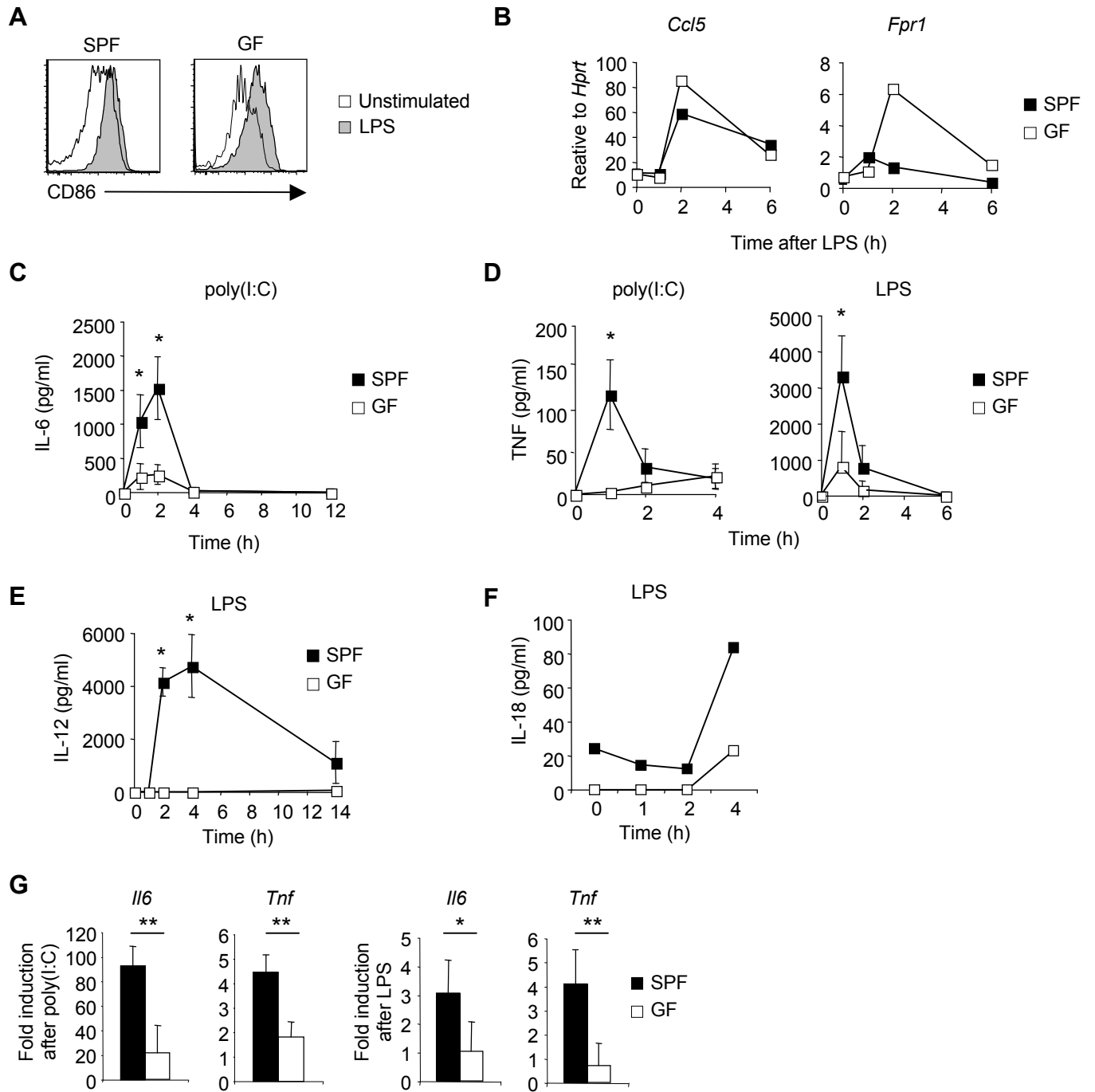


Figure 6 (Ganal et al.)

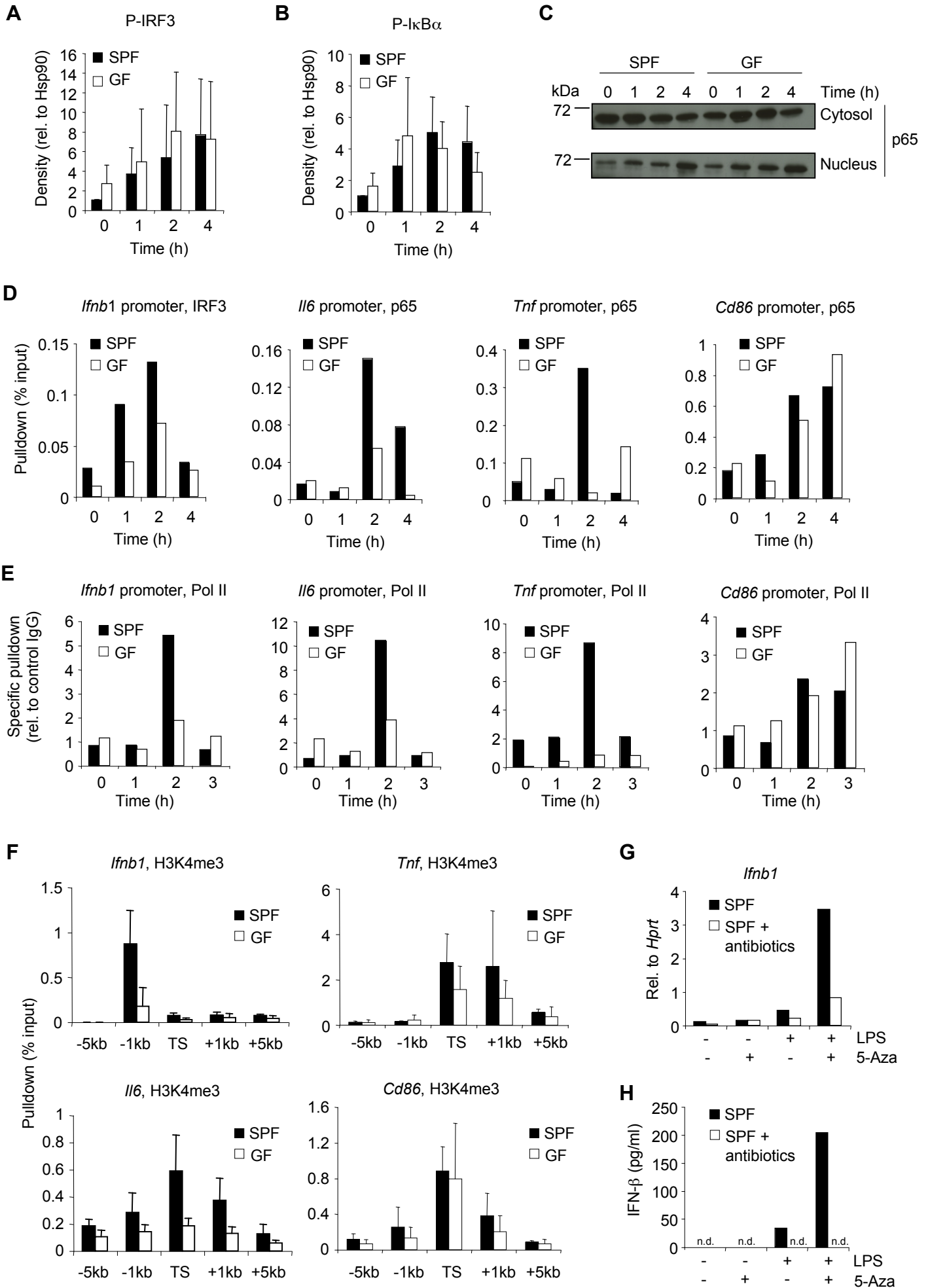


Figure 7 (Ganal et al.)

Supplemental Information

Permissive signals from the commensal microbiota are required for infection-induced type I interferon gene expression by non-mucosal mononuclear phagocytes

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Inventory of Supplemental Information

Figure S1: Supplement to Figure 1

Figure S2: Supplement to Figure 2

Figure S3: Supplement to Figure 4

Figure S4: Supplement to Figure 5

Figure S5: Supplement to Figure 6

Figure S6: Supplement to Figure 7

Supplemental Experimental Procedures

Supplemental References

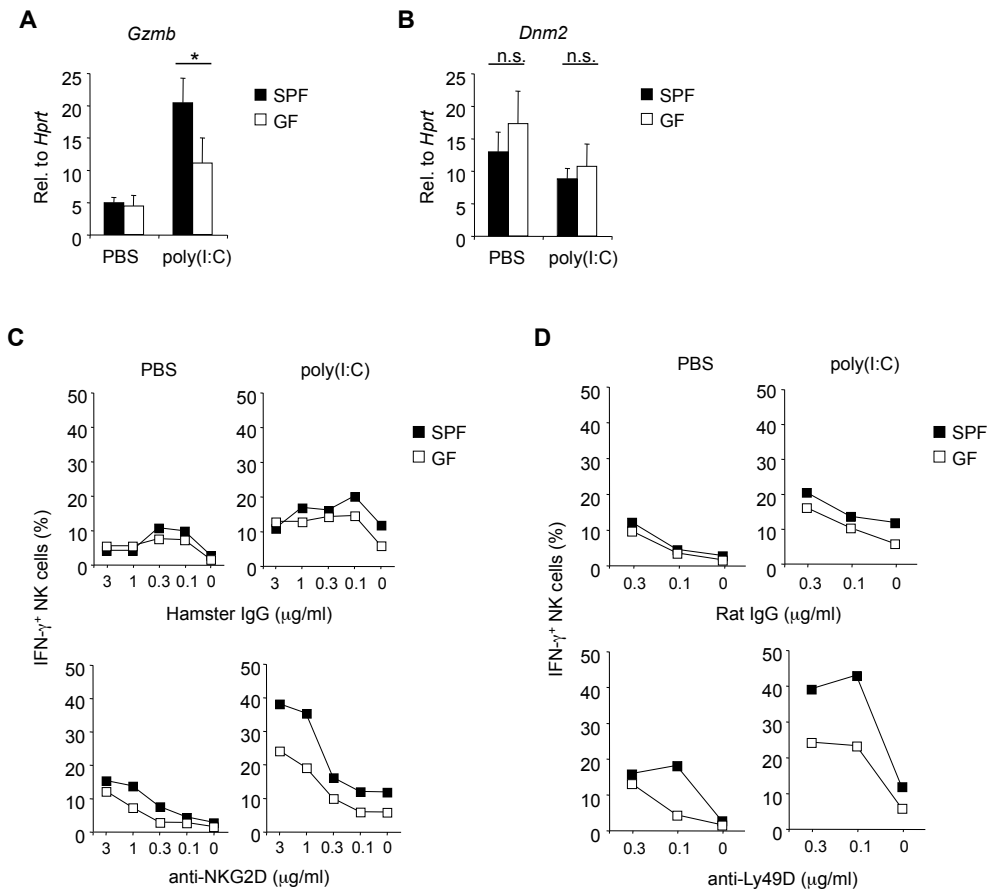


Figure S1: Supplement to Figure 1

(A,B) Groups of mice were injected with poly(I:C) or PBS. After 14 h, splenic NK cells were isolated by flow cytometry-based cell sorting and RNA was isolated. Expression of Granzyme B (*Gzmb*) (A) and Dynamin 2 (*Dnm2*) (B) was analyzed using qRT-PCR. Data represent mean (\pm SD, n=3).

(C,D) Groups of mice were injected with poly(I:C) or PBS. After 16 h, splenic NK cells were isolated and subjected to *in vitro* overnight stimulation with plate-bound anti-NKG2D (C), anti-Ly49D (D) or control antibodies. IFN- γ production by NK cells was determined by intracellular staining and flow cytometry.

Data are representative of two (C,D) or three (A,B) independent experiments. * $p \leq 0.05$, n.s. = not significant.

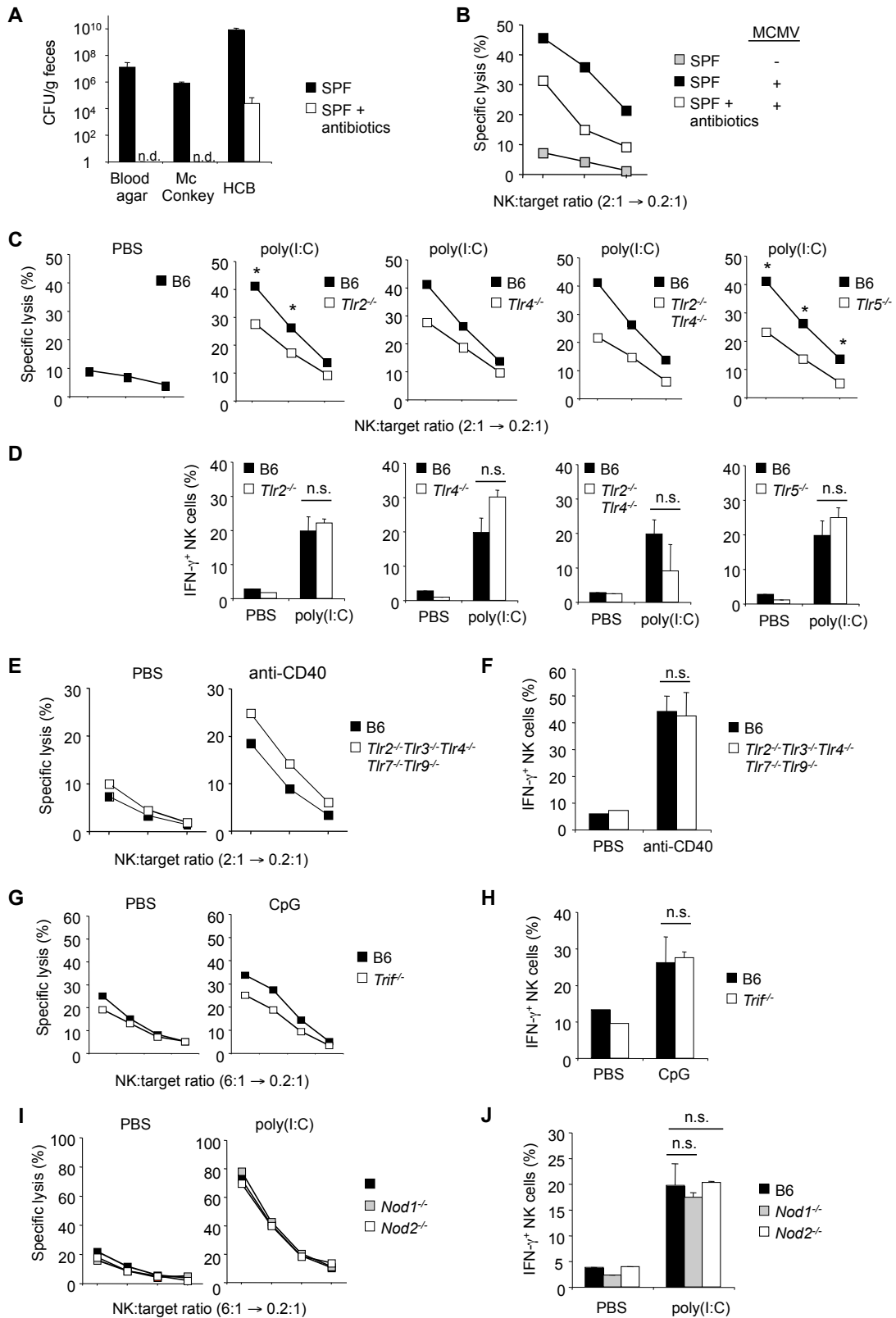


Figure S2: Supplement to Figure 2

(A) Mice were treated for 10 days with a cocktail of antibiotics. Serial dilutions of fecal samples were plated on blood agar, McConkey agar and glucose yeast extract cystein agar

(HCB). Colony forming units per gram of feces were determined after 24 h of aerobic culture (blood agar, McConkey) or 48 h of anaerobic culture (HCB). Data represent mean (\pm SD, n = 4). n.d. = not detectable.

(B) Groups of mice were infected i.v. with 1×10^5 pfu MCMV. Cytotoxic activity of splenic NK cells against YAC-1 target cells was determined 48 h after infection in a standard ^{51}Cr release assay.

(C-J) Groups of mice were injected with the indicated stimuli. Cytotoxicity (C,E,G,I) and IFN- γ production (D,F,H,J) in response to YAC-1 target cells were analyzed 14-16 h later. Data represent mean (\pm SD, n=3). * $p \leq 0.05$, n.s. = not significant.

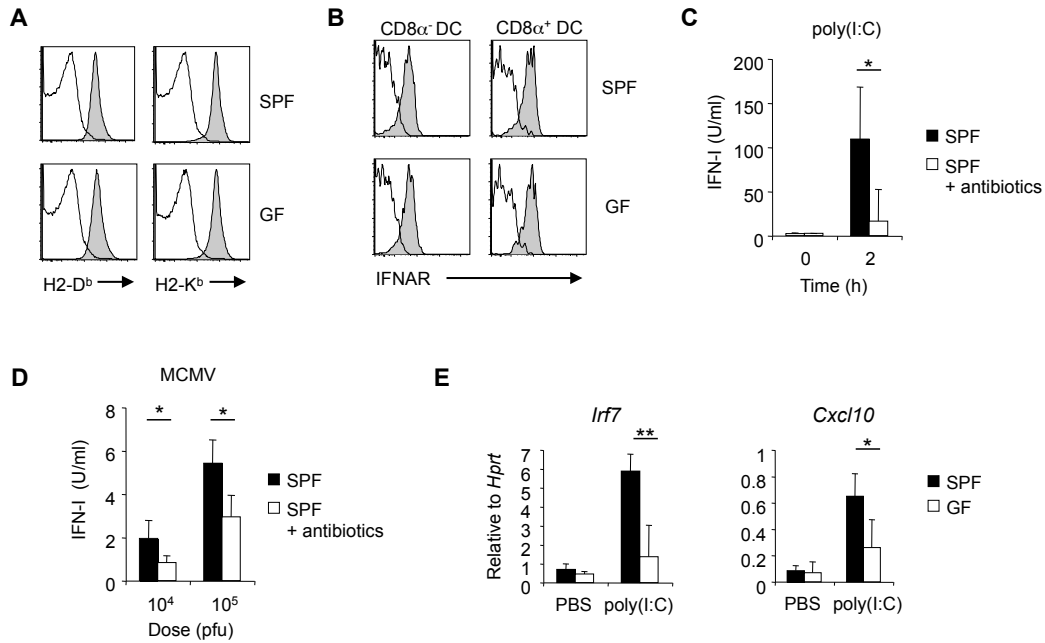


Figure S3: Supplement to Figure 4

(A) Splenocytes were stained with H2-D^b and H2-K^b (grey) antibodies or isotype control (open).

(B) IFNAR (grey) expression by CD11c⁺ MHCII⁺ CD8α⁻ or CD11c⁺ MHCII⁺ CD8α⁺ DC was determined by flow cytometry. Isotype control staining is shown as a comparison (open).

(C) SPF and antibiotic-treated SPF mice were injected with poly(I:C). Serum IFN-I concentrations were determined by VSV bioassay at the indicated time points. Data represent mean (\pm SD, n = 3).

(D) SPF and antibiotic-treated SPF mice were infected i.v. with different doses of MCMV. Serum IFN-I concentrations were determined by VSV bioassay at the indicated time points. Data represent mean (\pm SD, n = 4).

(E) *Irf7* and *Cxcl10* expression in spleen were determined by qRT-PCR 12 h after PBS or poly(I:C) injection.

Data are representative of two independent experiments. * p \leq 0.05, ** p \leq 0.01.

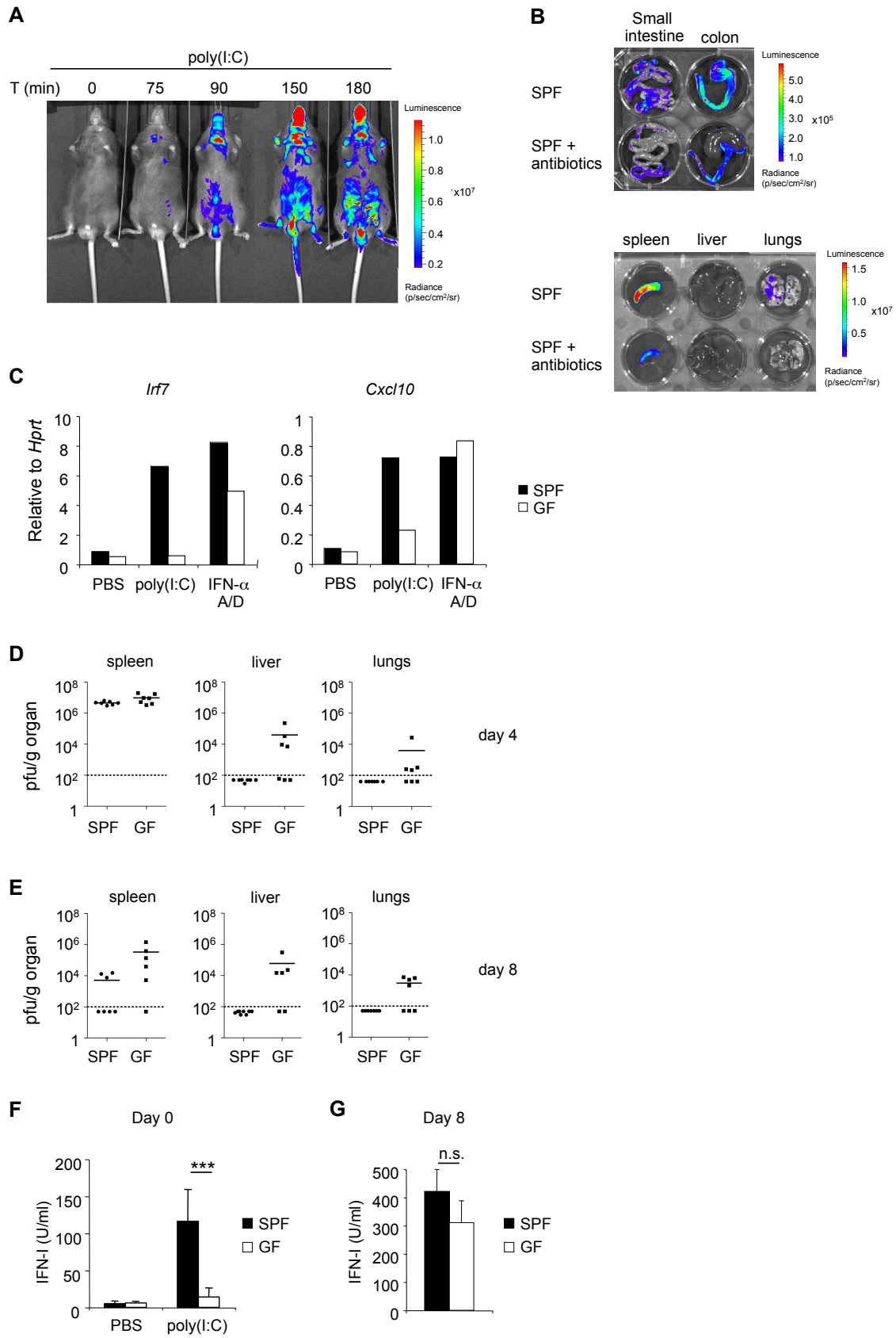


Figure S4: Supplement to Figure 5

(A,B) *Ifnb1*^{+Luc} mice were stimulated with poly(I:C) and luciferase activity was determined using a bioluminescence camera at the indicated time points (A). Luciferase activity in the indicated organs was determined 120 min post stimulation (B). Data from one representative mouse is shown.

(C) Groups of mice were injected with poly(I:C), human IFN- α A/D or PBS (control). *Irf7* and *Cxcl10* expression in the spleen was determined by qRT-PCR 12 h post stimulation.

(D-G) Groups of SPF and GF mice were infected i.v. with 200 pfu LCMV WE. A standard LCMV plaque assay was used to assess viral titers in spleen, liver and lungs on day 4 (D) and day 8 (E) post infection (n \geq 6). Serum IFN-I was determined in uninfected mice at day 0 (F) and day 8 (G), 2 h after PBS or poly(I:C) injection. Data show mean (\pm SD, n = 3).

Data are representative of two (D-G) or three (A-C) independent experiments.

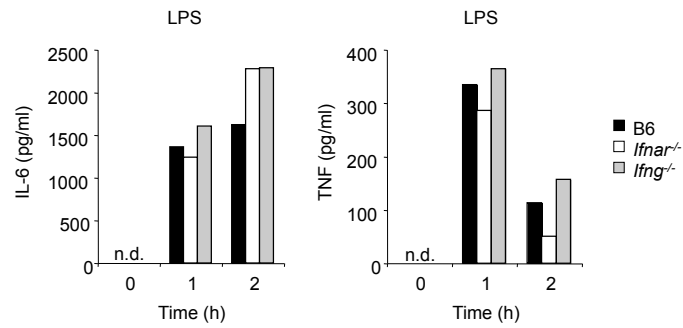


Figure S5: Supplement to Figure 6

B6, *Ifnar*^{-/-} and *Ifng*^{-/-} mice were injected with LPS. At the indicated time points, serum concentrations of IL-6 (left) and TNF (right) were determined by ELISA. Data represent the mean (n=2).

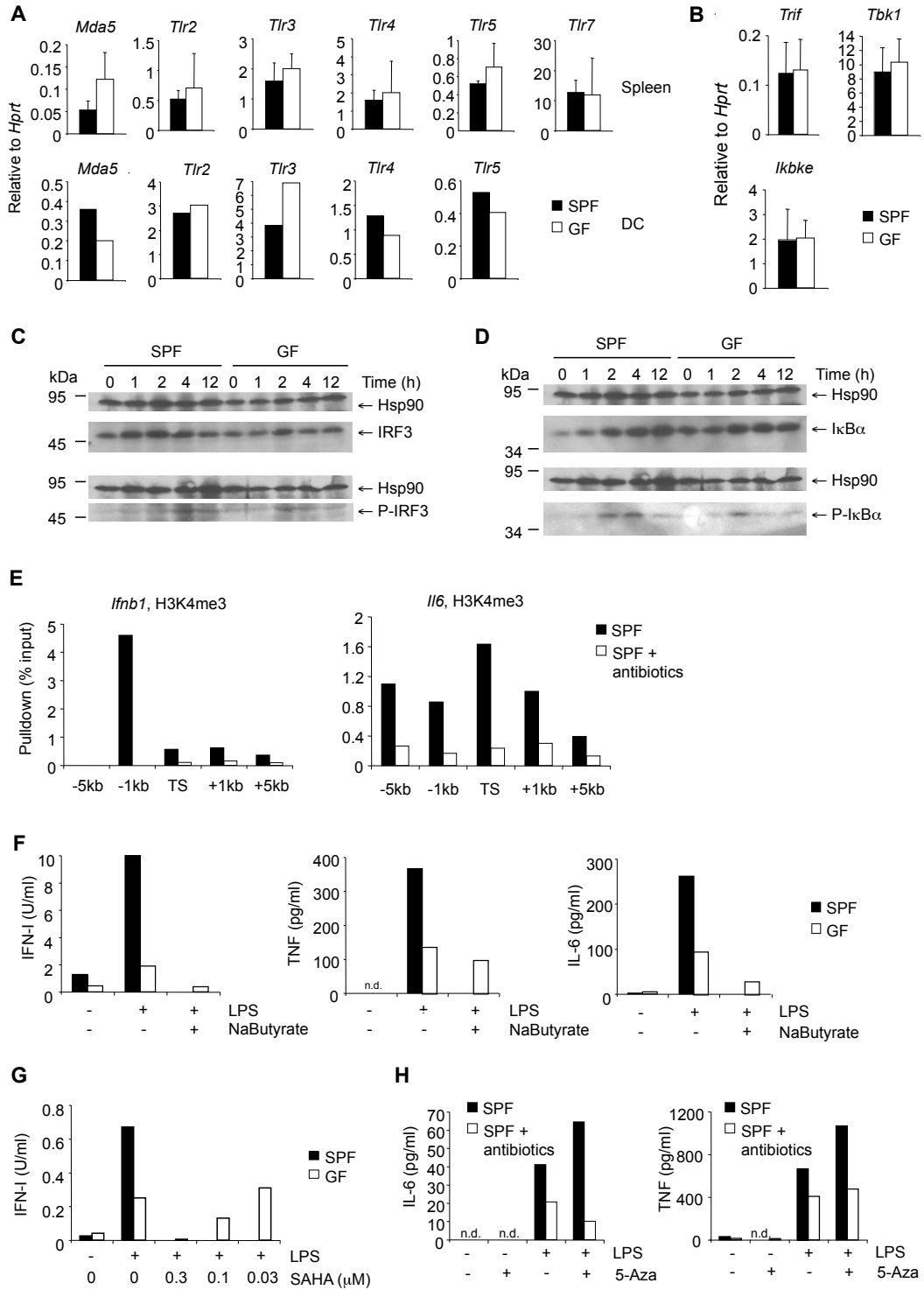


Figure S6: Supplement to Figure 7.

(A,B) Expression of the indicated genes in the spleen or by purified DC was determined using qRT-PCR. Data represent mean (\pm SD, $n \geq 5$).

(C,D) Groups of mice were injected with poly(I:C) and splenic DC were purified at the indicated time points. Western blot analysis of IRF3 (C) and phosphorylated IRF3 or I κ B α and phosphorylated I κ B α (D) in DC lysates.

(E) DC were purified from SPF and antibiotic-treated mice. H3K4me3 ChIP was performed and specific pulldown was determined around the transcriptional start (TS) sites of the indicated genes. Results represent specific pulldown relative to a DNA input sample. Pulldown with a control antibody was significantly lower or absent.

(F,G) Purified DC from SPF and GF mice were treated with the indicated HDAC inhibitors for 6 h and stimulated *in vitro* with LPS for 12 h. IFN-I concentration in the culture supernatants (F,G) was determined by VSV assay. IL-6 and TNF concentrations (F) were analyzed by ELISA.

(H) Groups of mice were treated *in vivo* with 5-azacytidine (5-Aza) or left untreated. Subsequently, splenic DC were isolated and stimulated for 4 h *ex vivo* in the presence of LPS. The concentrations of the indicated cytokines in culture supernatants were determined by ELISA.

Data are representative of two (F,G,H) or four (A-D) independent experiments. n.d. = not detectable.

Supplemental Experimental Procedures

Mice

B6-Ly5.2/Cr (CD45.1⁺), *Ifnar*^{-/-} (Muller et al., 1994), *Ifng*^{-/-} (Dalton et al., 1993), *Myd88*^{+/-}, *Myd88*^{-/-} (Adachi et al., 1998), *Myd88*^{-/-}*Trif*^{-/-}, *Tlr2*^{-/-} (Takeuchi et al., 1999), *Tlr4*^{-/-} (Hoshino et al., 1999), *Tlr2*^{-/-}*Tlr4*^{-/-}, *Tlr2*^{-/-}*Tlr3*^{-/-}*Tlr4*^{-/-}*Tlr7*^{-/-}*Tlr9*^{-/-} (Conrad et al., 2009), *Tlr5*^{-/-} (Uematsu et al., 2006), *Nod1*^{-/-} (Chamaillard et al., 2003), *Nod2*^{-/-} (Kobayashi et al., 2005), *Trif*^{-/-} (Hoebe et al., 2003) and *Ifnb1*^{+Luc} (Lienenklaus et al., 2009) mice, all on a B6 background, were bred in our animal facilities. *Tlr2*^{-/-}, *Tlr4*^{-/-}, *Tlr2*^{-/-}*Tlr4*^{-/-} and *Tlr5*^{-/-} were a kind gift from Marina Freudenberg and Stefan Martin (Freiburg). *Nod1*^{-/-} mice were generously provided by Birgitta Henriques-Normark (Stockholm). *Ifnb1*^{+CD11c-luc} were obtained by crossing IFN- β ^{+floxB-luc} (Solodova et al., 2011) mice with CD11c-Cre^{Tg} mice (Caton et al., 2007) and *Ifnb1*^{+LysM-luc} mice by crossing IFN- β ^{+floxB-luc} with LysM-Cre^{Tg} mice (Clausen et al., 1999). NMRI mice colonized with Altered Schaedler's Flora and germ-free NMRI mice were housed in our animal facilities. All mice were used at 8–14 weeks of age. Experiments were approved by, and were in accordance with, local animal care and use committees (Regierungspräsidium Freiburg).

Antibiotic treatment

For eradication of the commensal microbiota, mice were provided with drinking water containing 1 mg/ml Cefoxitin (Santa Cruz Biotechnology), 1 mg/ml Gentamicin (Sigma), 1 mg/ml Metronidazol (Sigma), and 1 mg/ml Vancomycin (Hexal). Antibiotics were renewed every other day. Cages were changed daily throughout the course of treatment. Feces were plated in serial dilutions on Colombia blood agar, McConkey agar and glucose yeast extract cysteine agar (HCB, for growth of anaerobic bacteria) in order to track the success of the treatment.

Recolonization of germ-free mice

Germ-free animals were cohoused in the same cage as conventional (SPF) mice for at least 7 days.

***In vivo* and *in vitro* microbial stimulations**

For *in vivo* stimulation, 50 µg ultrapure LPS from *E. coli* O111:B4 (Invivogen), 100 µg poly(I:C) (Sigma), 100 µg CpG1668 (Invitrogen), 75 µg anti-CD40 (clone FGK4.5, Bio Excel) or 1×10^5 U human recombinant IFN- α A/D (Roche) were prepared in 250 µl PBS and injected intraperitoneally. Purified myeloid cell populations were stimulated *in vitro* with either 0.1 or 1 µg/ml LPS or 5 µg/ml poly(I:C) for the time periods indicated.

***In vitro* treatment with HDAC inhibitors**

Isolated DC were cultured for 6 h in the presence of HDAC inhibitors (sodium butyrate or suberoylanilide hydroxamic acid, SAHA) at different concentrations and subsequently stimulated with 0.1 µg/ml LPS for 12 h.

***In vivo* treatment with 5-azacytidine**

Mice were intraperitoneally injected with 5 µg/g 5-azacytidine (Calbiochem) at 72 h, 48 h and 24 h before the start of the experiment. Subsequently, splenic DC were isolated and stimulated *ex vivo* in the presence of 0.1 µg/ml LPS for 4 h.

Determination of virus titers

MCMV titers were determined in a standard MCMV plaque assay (Brune et al., 2001). LCMV load in different organs was determined by plaque assay from organ samples on day 4 and day 8 post infection using a previously described method (Lucas et al., 2010).

***In vitro* stimulation of NK cells with plate-bound antibodies**

Round-bottom 96-well plates were coated with purified antibodies specific for NKG2D (clone A10, purified in our own laboratory) or Ly49H (clone 4E5, BD) diluted in PBS on a shaker at 4°C overnight. Splenic NK cells were isolated from mice primed with TLR ligands 18 h before or from control mice using the NK cell isolation kit and MACS (Miltenyi). 5×10^4 NK cells were seeded per well and incubated in the presence of 250 ng/ml human IL-2 for 12 h. IFN- γ production by NK cells was determined using intracellular staining and flow cytometry.

VSV bioassay

Serial dilutions (1:2) of serum samples were prepared in duplicate in 96-well flat-bottom plates (Nunc) containing 50 μ l DMEM 5% FCS. Murine IFN- β (PBL Interferonsource) was used as an internal standard (highest standard: 100 U/ml). 1.5×10^4 L929 cells in 50 μ l medium were added to each well and the plates incubated over night at 37°C before 50 μ l of vascular stomatitis virus (VSV) were added at a final concentration of 3×10^5 pfu/ml. A positive control contained L929 cells but no VSV, a negative control contained L929 cells incubated without protective type I IFN plus VSV. Cells were grown in the presence of the virus for 48 h at 37°C. Cells that have previously been treated with type I IFNs are protected against lysis by the VSV. MTT (Thiazolyl Blue Tetrazolium Blue, Sigma) was added to each well to obtain a final concentration of 1 mg/ml and the samples incubated for 6-8 h at 37°C. Since living cells are able to reduce yellow MTT into a blue product, this assay allows for the quantification of the amount of living cells per well. Cells were lysed with 100 μ l 10% SDS/0.01N HCl at 37°C over night and absorbance at 550 nm was measured using an ELISA-Plate-Reader Spectra max 340 (MolecularDevices). The mean absorbance values of the standard were related to the negative control (100% protection of L929 cells, all cells alive) and plotted against the known standard concentration. A cellular protection of 50%

corresponds to 1 U/ml and the factor describing the difference between determined and actual concentration of the standard at 50 % protection was defined as arbitrary units. Sample values were treated in the same way and plotted against the corresponding dilution factor. The dilution factor resulting in 50% protection was determined (df_{50}). The sample concentrations were calculated according to the following formula: $c \text{ (U/ml)} = df_{50} \times \text{arbitrary units}$.

Cell isolation and flow cytometry

Splenocytes were isolated and stained for cell surface markers as previously described (Vonarbourg et al., 2010). For intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Bioscience) and subsequently stained in saponin buffer (PBS, 2.5 mM EDTA, 2.5% FCS, 0.5% w/v Saponin) with antibodies specific for intracellular antigens. Cells were washed twice with saponin buffer and analyzed using a FACS CantoII flow cytometer (BD Bioscience). The indicated sorted cell populations were highly purified (for $\geq 98\%$ purity) using a MoFlo cell sorter (Beckman Coulter).

Antibodies and staining reagents

Fluorophore-conjugated antibodies specific for the indicated mouse antigens were purchased from eBioscience, unless stated differently: CD3 ϵ (145-2C11), CD8 α (53-6.7), CD11b (M1/70), CD11c (N418), CD19 (1D3), CD27 (LG.7F9), CD45.1 (A20), CD45.2 (104), CD44 (IM7), CD49b (DX5), CD62L (MEL-14), CD86 (GL1), CD122 (5H4), F4/80 (A3-1), Granzyme B (NGZB), H2-D^b (28-14-8, BD Biosciences), H2-K^b (AF6-885, BD Biosciences), IFN- γ (XMG1.2), IL15R α (polyclonal, R&D Systems), KLRG-1 (2F1), NK1.1 (OK136), NKG2AB6 (16a11), NKG2D (CX5), NKp46 (29A1.4), Ly49A (A1), Ly49C/I (5E6), Ly49D (4E5), Ly49G2 (Cwy-3), Ly48H (3D10), MHC class II (M5/114.5.2).

Matched antibody pairs for ELISA were purchased from R&D Systems: anti-IL-12 (MAB419), biotinylated anti-IL-12 (BAF419), anti-IL-6 (MAB406), biotinylated anti-IL-6 (BAF406), anti-TNF (AF-410-NA), biotinylated anti-TNF (BAF410). Avidin-HRP (eBioscience) was used for detection.

Primary antibodies for western blot analysis were purchased from Cell Signaling Technology: anti-IRF-3 rabbit mAb (D83B9), anti-phospho-IRF-3 rabbit mAb (4D4G), anti-I κ B α (L35A5) and anti-phospho-I κ B α mouse mAb (5A5). Anti-p65 (C-20) was from Santa Cruz Biotechnology. Secondary antibodies anti-rabbit-HRP and anti-mouse-HRP were purchased from Jackson ImmunoResearch Laboratories. Anti-RNA Polymerase II rabbit antibody (H-224), anti-p65 (C-20) and anti-IRF3 (FL-425) for ChIP were purchased from Santa Cruz Biotechnology. Anti-H3K4me3 antibody (ab8580) was from abcam. Control rabbit IgG was purchased from Jackson ImmunoResearch Laboratories.

Real-time PCR

Real-time PCR was performed as previously described (Kiss et al., 2011). The amount of mRNA was normalized to that of the housekeeping gene *Hprt1* (encoding hypoxanthine guanine phosphoribosyl transferase).

The following Taqman probes were used:

Cxcl10 (Mm00445235_m1),

Gzmb (Mm00442834_m1),

Hprt (Mm00446968_m1),

Ifnb1 (Mm00439546_s1),

Ifna4 (Mm00833969_s1),

Il15 (Mm00434210_m1),

Irf7 (Mm00516788_m1),

Mda5 (Mm_00459183).

Primer sequences for gene expression analysis

Gene of interest	Forward primer	Reverse primer
<i>Ccl5</i>	ATATGGCTCGGACACCACTC	TCCTTCGAGTGACAAACACG
<i>Dynamin2</i>	TTCGGGTCTACTCACCACAC	CGGCACCTTAGTGATGCCTG
<i>Fpr1</i>	CCATTTGGTTGGTTCATGTGC	CTTCTTGGCTAGGCTCACAGT
<i>Ikbke</i>	CTGGATGTCCCAAAGTTCGT	GCTCCACTGCTGAACCTTTC
<i>Il6</i>	TCGGAGGCTTAATTACACATGTTCT	GCATCATCGTTGTTTCATAAATCA
<i>Tbk1</i>	GAGTACCTGCATCCGGACAT	AGTGCCTGAAGACCCTGAGA
<i>Trif</i>	CACTGCCTCCAGTCTCTTCC	GATCAGTCAGAGGGCCCATATA
<i>Tlr2</i>	AGCATCCTCTGAGATTTGACG	GGGGCTTCACTTCTCTGCTT
<i>Tlr3</i>	TCCCCAAAGGAGTACATTAGA	GATACAGGGATTGCACCCATA
<i>Tlr4</i>	CTGATCCATGCATTGGTAGGT	GGACTCTGATCATGGCACTG
<i>Tlr5</i>	CGGCAAGCATTGTTCTCC	CTGGAGCCGAGTGAGGTC
<i>Tlr7</i>	CGTGTCCACATCGAAAACAC	TGATCCTGGCCTATCTCTGAC
<i>Tnf</i>	TCTTCTCATTCTGCTTGTGG	GGTCTGGGCCATAGAACTGA

Protein lysates and Western blot

Sorted dendritic cells were lysed in NP40 buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton-X-100, 0.5% NP-40, complete protease and phosphatase inhibitor cocktail) for 30 min on ice. Protein concentration in the lysates was determined using the BCA Protein Assay (Thermo Scientific) and 50 µg of protein in Laemmli buffer (125mM Tris-HCl pH 6.8, 4% SDS, 10% beta-mercaptoethanol, 10% glycerol, 0.02% bromophenolblue) was loaded on a 15% polyacrylamide gel. Proteins were separated under voltage in 1x TGS electrophoresis buffer (25mM Tris base, 192 mM glycine, 0,1% w/v SDS). The peqGOLD Protein Marker IV

(PEQLAB) was used to determine protein sizes. The gel was blotted onto a nitrocellulose membrane (Whatman®) in transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol) using a wet/tank blotting system (Bio-Rad). The membrane was blocked in 5% v/w BSA in TTBS (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% Tween-20) on a shaker for 1 h at room temperature. Primary antibodies were diluted according to the manufacturer's protocol in 5% v/w BSA in TTBS and incubated at 4°C on a shaker over night. The blot was washed 3 times for 10 min with TTBS on a shaker at RT. Secondary antibodies were diluted as per the manufacturer's protocol in 5% v/w BSA in TTBS and incubated with the membrane for 1 h at RT. The blot was washed 3 times for 10 min with TTBS on a shaker at RT and developed using the SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Scientific) and Amersham Hyperfilm ECL (GE Healthcare) films.

Nuclear fractionation

Cells were lysed in hypotonic buffer (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, complete protease inhibitor cocktail) for 15 min on ice. NP-40 was added at a final concentration of 0.5% and samples were vortexed for 10 sec. After centrifugation (300 rpm, 10 min, 4°C), the supernatant, which contained the cytosolic fraction, was collected and nuclei were lysed in cell extraction buffer (100 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 0.1% SDS, complete protease and phosphatase inhibitor cocktail) for 30 min on ice, with vortexing occurring at 10 min intervals. Lysates were cleared by centrifugation (14000 x g, 30 min, 4°C) and collected as nuclear fraction. Purity of the fractions was controlled in western blot using antibodies against GAPDH (cytosolic marker) and histone H4 (nuclear marker).

Chromatin immunoprecipitation (ChIP)

Splenic DC were isolated using CD11c beads (Miltenyi). DC (3×10^6) were stimulated *in vitro* with 0.1 $\mu\text{g/ml}$ LPS for the indicated time. Cells were washed with PBS and cross-linked with 1% formaldehyde for 8 min at room temperature. Cross-linking was halted by adding glycine at a final concentration of 0.125 mM. For RNA Polymerase II ChIP, cells were washed twice (PBS, 1 mM NaF, 1 mM NaVO_4 , 20 mM Na-butyrate) and resuspended in 700 μl lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% TritonX-100, 0.1% Na-deoxycholate, 20 mM Na-butyrate and complete protease inhibitor cocktail). The lysates were sonicated (3 x 9 s) to obtain DNA fragments of 300-1000 bp. The cells were then centrifuged and the supernatant collected. The supernatant was incubated overnight at 4°C on a rotating wheel with protein G Dynabeads® (Invitrogen) coated with either 4 μg anti-RNA Polymerase II antibody (SantaCruz Technology) or control rabbit IgG. Elution, de-crosslinking and DNA purification were carried out as previously described (Kiss et al., 2011).

For ChIP of transcription factors and histone modifications, a modified protocol was applied. Cell membranes were lysed in 500 μl of buffer L1 (50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% NP-40, 10% glycerol, complete protease inhibitor cocktail) and kept for 5 min on ice. Nuclei were pelleted (3000 rpm, 5 min, 4°C) and lysed in 500 μl of buffer L2 (50 mM Tris, pH 8.0, 5 mM EDTA, 1% SDS, complete protease inhibitor cocktail). The lysates were sonicated (3 x 9 s) to obtain DNA fragments of 300-1000 bp. The cells were centrifuged and the supernatant was collected. For each pulldown, 150 μl of lysate were diluted 1:10 with dilution buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 200 mM NaCl, 0.5% NP-40) and precleared with salmon-sperm DNA-saturated Protein A-agarose beads for 1 h at 4°C. The precleared lysate was incubated with 2 μg of antibody rotating overnight at 4 °C. Chromatin pulldown with salmon-sperm DNA-saturated Protein A-agarose beads was performed for 1 h

at 4°C. Beads were then washed 4 times for 5 min on ice with wash buffer (20 mM Tris pH 8.0, 2 mM EDTA, 0.1% SDS, 1% NP-40, 500 mM NaCl) and 4 times for 5 min on ice with TE buffer. Elution of DNA was performed with 3 x 50 µl of elution buffer (TE buffer, 2% SDS) at room temperature and reverse cross-linked at 65°C overnight. DNA purification was carried out a previously described (Kiss et al., 2011). The purified DNA underwent a ten-fold dilution and RT PCR was performed on 4.5 µl of the diluted sample using the SYBR® Green Master Mix (Applied Biosystems) in conjunction with the ABIPrism 7900 sequence detector (Applied Biosystems). An input DNA sample was taken before pulldown and included in the RT-PCR as a reference value. Results represent percent pulldown relative to input and were calculated according to the following formula: $\text{Pulldown (\% input)} = 2^{-(CT_{\text{pulldown}} - CT_{\text{input}})} \times 100$. Percent pulldown with the specific antibody was normalized to percent pulldown with control IgG.

Primer sequences for ChIP

Gene of interest	Forward primer	Reverse primer
<i>Cd86 promoter</i> (<i>RNA Pol II, p65</i>)	TGATGGGAGTAGATGCAGAGG	GCCCTCTTCTAAGGGGTTC
<i>Cd86 -1kb</i>	TGCACTTGTCTCTGACTCTTGTC	TCGAGGCTAGACAAGACAACC
<i>Cd86 -5kb</i>	TCCCCTATCATTTCCTGAG	GGTGGGCAAAAAGAGTGAAA
<i>Cd86 TS</i>	AAAGTCAACCACCAGGGTACA	TGCCTACAGGAGCCACTCTT
<i>Cd86 +1kb</i>	CAGATTCTGAGGGGTGACTG	TCCCATTGCCTATTTGTGTCT
<i>Cd86 +5kb</i>	ACGCATAGCCACACACAAAT	GCTGGTAAATGTGGGGAGAG
<i>Ifnb1 promoter</i> (<i>IRF-3</i>)	GAGGAAAAGTGAAGGGAGAAGT	GAGGCAAAGGCTGTCAAAG
<i>Ifnb1 promoter</i> (<i>RNA Pol II, TS</i>)	ATAGCACAGGCCATGAAGGAAGAT	TGGAGAAGCACAGCAGGAACG
<i>Ifnb1 -1kb</i>	CCAATGGAGATTTCTCCAAA	CTGCAGTGCTCTCATGCAA
<i>Ifnb1 +1kb</i>	AATGGGTTGGTGGTTACCTG	AACTCCCCGAGACCATTACC
<i>Ifnb1 -5kb</i>	TGAGTTCTCCAGCCCTTGAT	TACCCTAAGGGCGGTGTATG
<i>Ifnb1 +5kb</i>	AGGGAAATGACTGTGGCATC	AGCAGAAGGTACCCAGGTT

<i>Il6 promoter</i> (<i>RNA Pol II, p65, TS</i>)	TCGATGCTAAACGACGTCAC	ACTCATGGGAAAATCCCACA
<i>Il6 -1kb</i>	GGTGGACAGAAAACCAGGAA	TAACCCCTCCAATGCTCAAG
<i>Il6 +1kb</i>	AGAGGCCAGCTTGGTCTACA	GGTAGGGTTGGTTGGGTTTT
<i>Il6 -5kb</i>	TAGGCACCAAACCTCCAAAC	TTCCGTATCCAGGAAACCAG
<i>Il6 + 5kb</i>	TCATGCAGTGTGGGAAAGAG	TCTGAGCAGCTGTCTGGAAA
<i>Tnf promoter</i> (<i>p65</i>)	GGCTGAGTTCATTCCCTCTG	CTTGGAGGAAGTGGCTGAAG
<i>Tnf promoter</i> (<i>RNA Pol II, TS</i>)	AGCTTTTCCCCGCCCTCTTCC	CCTCCTGGCTAGTCCCTTGCTGTC
<i>Tnf -1kb</i>	CTCCCAGAGACATGGTGGAT	TCATCTCTCTCCACCCTTGG
<i>Tnf -5kb</i>	CCTGTTCCAGGAGAGCTGAC	AAGTGCCACAGGCCACAT
<i>Tnf +1kb</i>	AAGATGGAGGAAGGGCAGTT	GATCCTGGAGGGGAAGAGAC
<i>Tnf +5kb</i>	CCCCTTAAAGAAGGGCTGTC	TAGTCCTGGGTCACCTGGAA

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