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

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SUMMARY

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 in response to microbial stimulation, mononuclear phagocytes residing in nonmucosal lymphoid organs of germ-free mice failed to induce expression of a set of inflammatory response genes, including those encoding the various type I interferons (IFN-I). Consequently, NK cell priming and antiviral immunity were severely compromised. Whereas pattern recognition receptor signaling and nuclear translocation of the transcription factors NF-κB 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.

INTRODUCTION

Mucosal surfaces of multicellular organisms are constantly exposed to a complex ecosystem of commensal bacteria. It is believed that millions of years of coevolution 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 nonmucosal 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 nonmucosal 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 (e.g., dendritic cells [DCs], 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 costimulatory molecules and soluble factors required to prime and coordinate adaptive immunity (Janeway, 1989). In nonmucosal lymphoid organs (such as spleen and peripheral lymph nodes), the total numbers of macrophages (Bauer et al., 1963; Mørland 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 antimicrobial activity of macrophages from germ-free mice may be reduced (Mitsuyama et al., 1986;

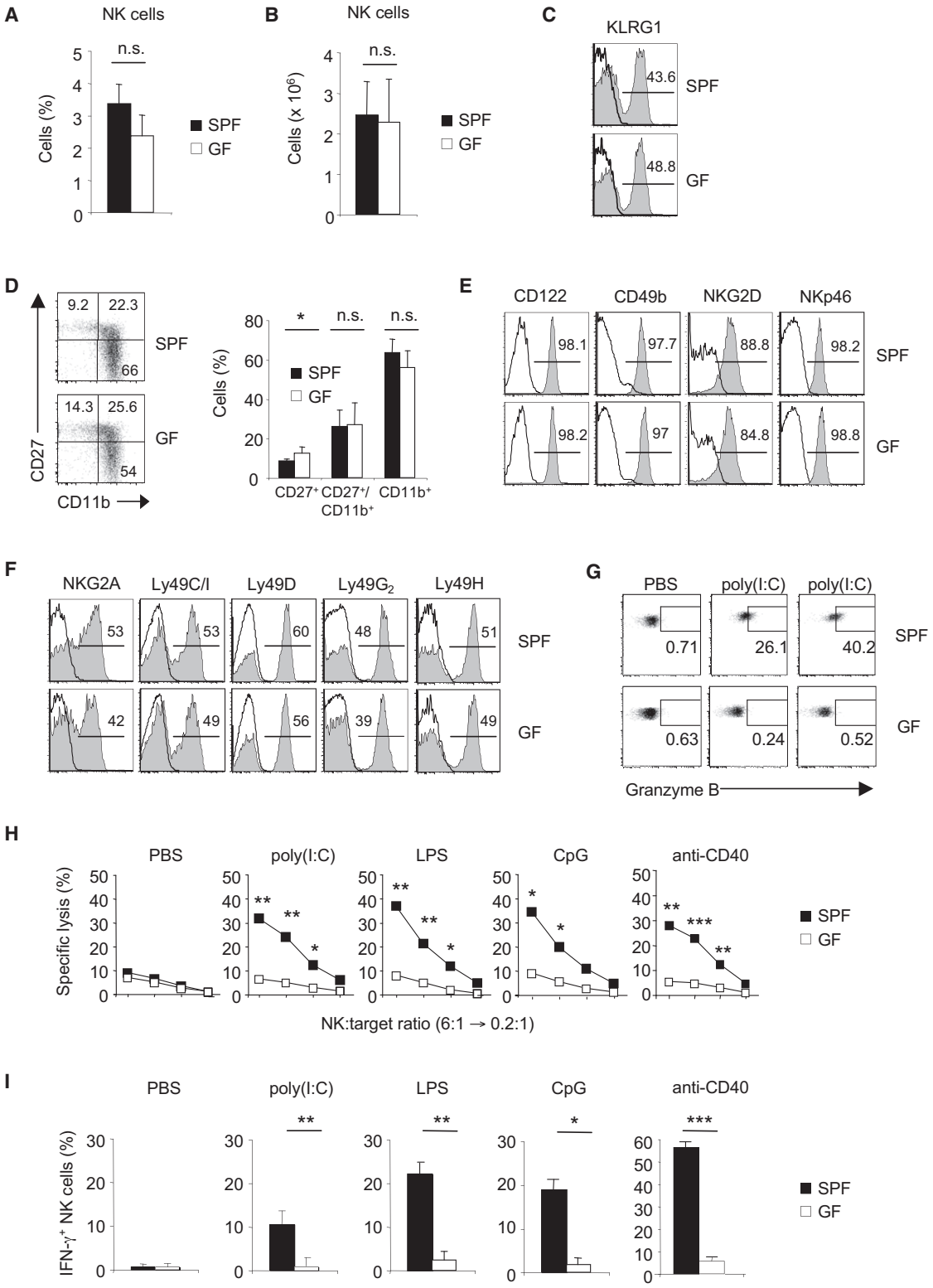

Figure 1. Impaired NK Cell Responses after Microbial Stimulation in Germ-free Mice

(A and 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 (±SD, n ≥ 5) and absolute numbers (±SD, n ≥ 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 (gray) or isotype control antibody (open) of NK1.1$^+$CD3$^-$ cells. Numbers represent percent KLRG1$^+$ cells.

Mørland 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 3 and 7 (IRF3 and IRF7). Activated IRF3 and IRF7 induce the transcription of type I interferons (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 DCs induce cell-mediated immunity by NK cells and $CD8^+$ T cells mainly through the induction of IL-15 expression and its *trans*-presentation by DCs to lymphoid cells (Lucas et al., 2007; McCartney et al., 2009). The role of commensal microbiota in the production of IFN-I is unclear. Although 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 DCs 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 (Huh et al., 1981) differences 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 nonmucosal lymphoid organs of germ-free mice could not be primed to mount effective antiviral 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 DCs 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-κB 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 nonmucosal sites, probably 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 NK Cell Function

We analyzed phenotype and function of NK cells residing in nonmucosal 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 coexpressing CD11b and CD27 (Hayakawa and Smyth, 2006). KLRG1 expression by NK cells was not reduced in germ-free mice (Figure 1C). Although coexpression 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 coexpressed the β chain of the IL-2 and IL-15 receptors (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

(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 (±SD, n = 7) of NK cells in each subset.
(E and 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 costaining with the indicated marker (gray) 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 hr 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 and 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 hr 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 (±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.

and SPF mice (Figure 1F). Thus, commensal bacteria are not required for the development, differentiation, or 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–1I; Figure S1A available online). Similar results were obtained when NK cell activity was primed by injecting CD40 antibodies to directly trigger mononuclear phagocytes (Figures 1H and 1I). Whereas 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- to 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 antibiotic-treated mice were infected with 10^4 or 10^5 plaque-forming units (pfu) of MCMV (Smith strain). After infection, NK cell activity in antibiotic-treated mice was substantially reduced (Figures 2C, 2D, and S2B). Although 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 recolonized 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 recolonization. Our data from recolonization 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 nonmucosal 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 nonmucosal organs. In mice genetically lacking single TLR or combinations of up to five TLRs (namely TLR2, TLR3, TLR4, TLR7, TLR9) (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, 2J, and S2G–S2J). 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.

Priming of NK Cells Is Defective 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. 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 DCs (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 DCs, we transferred highly purified NK cells from germ-free (CD45.2) into SPF (CD45.1) mice and measured the activity of host and donor-derived NK cells after application of poly(I:C) (Figure 3A). Although NK cells could not be primed after injection of microbial ligands into germ-free mice (Figures 1G–1I), 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–1F), 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). Although 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

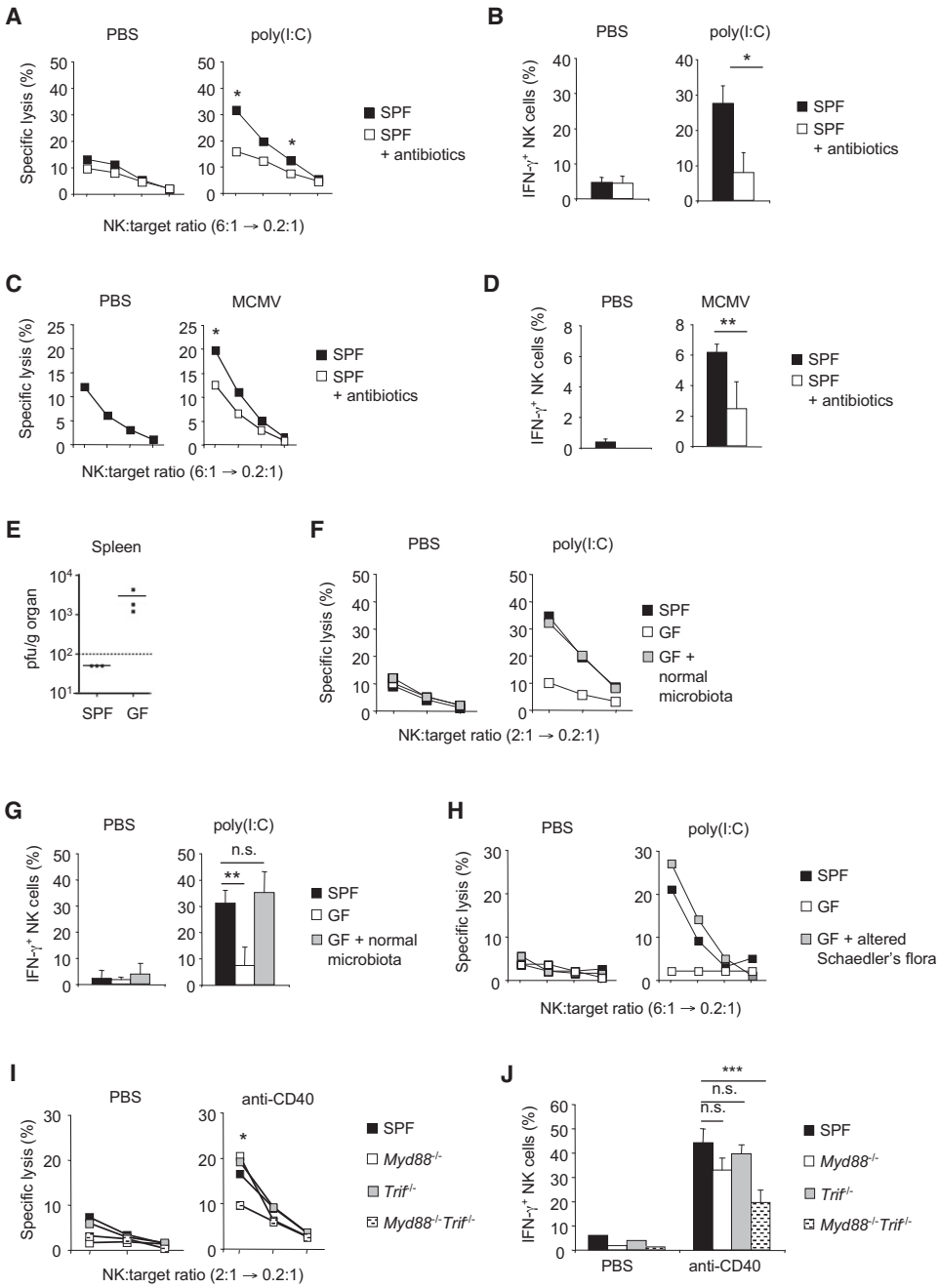

Figure 2. NK Cell Responsiveness Is Dynamically Regulated by the Commensal Microbiota

(A and 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 hr after stimulation. Data represent mean (±SD, n = 3).

(C and 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 hr after stimulation. Data represent mean (±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 and G) Groups of GF mice were recolonized 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 hr after stimulation. Data represent mean (±SD, n = 3).

(H) Groups of SPF, GF, and GF NMRI mice recolonized 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 hr later. Data represent mean specific lysis (n = 5).

(I and J) Cytotoxicity (I) and IFN-γ production (J) of splenic NK cells from the indicated mouse strains against YAC-1 targets 14–16 hr after stimulation with CD40 antibody. Data represent mean (±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.

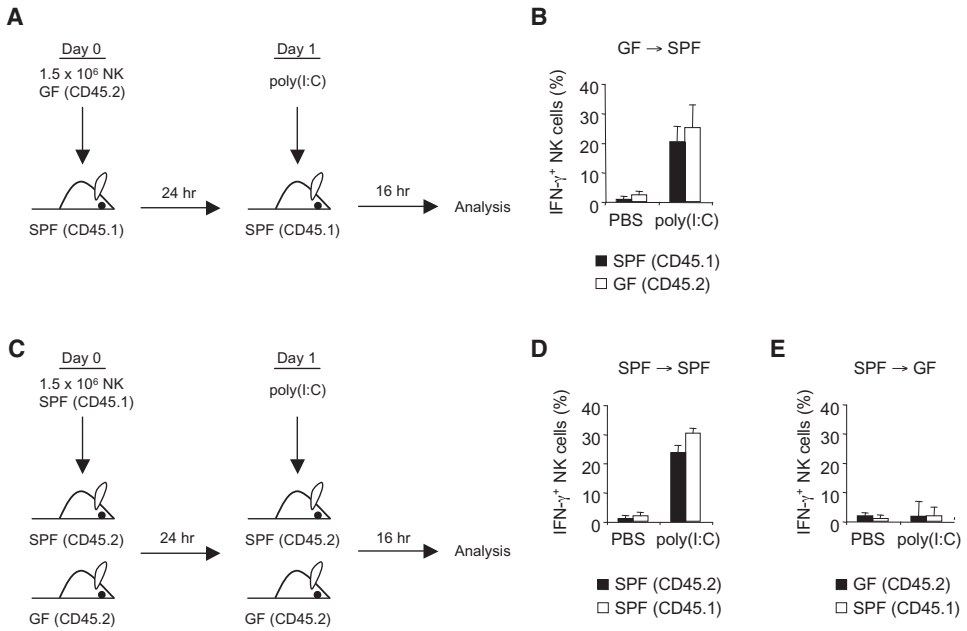

Figure 3. Failure of NK Cell Priming in Germ-free Mice

(A and B) Experimental design shown in (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) 1 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 hr. Data represent mean (±SD, n = 3).

(C–E) Experimental design shown in (C). Sorted splenic NK cells from SPF mice (CD45.1) were transferred into congenic (CD45.2) SPF (D) or GF (E) mice. IFN-γ production (D and E) by donor (CD45.1) or host-derived (CD45.2) splenic NK cells against YAC-1 targets was determined after 16 hr. Data represent mean (±SD, n = 3).

Data are representative of three independent experiments.

mice (Figure 3E). Thus, the functional defect of NK cells in germ-free mice is NK cell extrinsic and reflects deficient priming signals by bystander cells, most probably DCs 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 DCs *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 CD8α$^-$ and CD8α$^+$ DCs and macrophages (i.e., CD11b$^+$F4/80$^+$ cells), were comparable between SPF and germ-free mice (Figures 4A–4D). However, after stimulation with microbial ligands, DCs of germ-free mice did not show increased expression of *Il15* (Figure 4E) or IL-15Rα (Figure 4F). Thus, DCs and macrophages are normally represented in the absence of commensal bacteria in nonmucosal organs but may not provide NK cell priming signals.

Upregulation of IL-15Rα 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). Because IFNAR expression by DCs 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) or 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 upregulated after 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 nonmucosal (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$^+$ DCs (*Ifnb1*$^{+/CD11c\text{-}luc}$ mice) or in all macrophages and

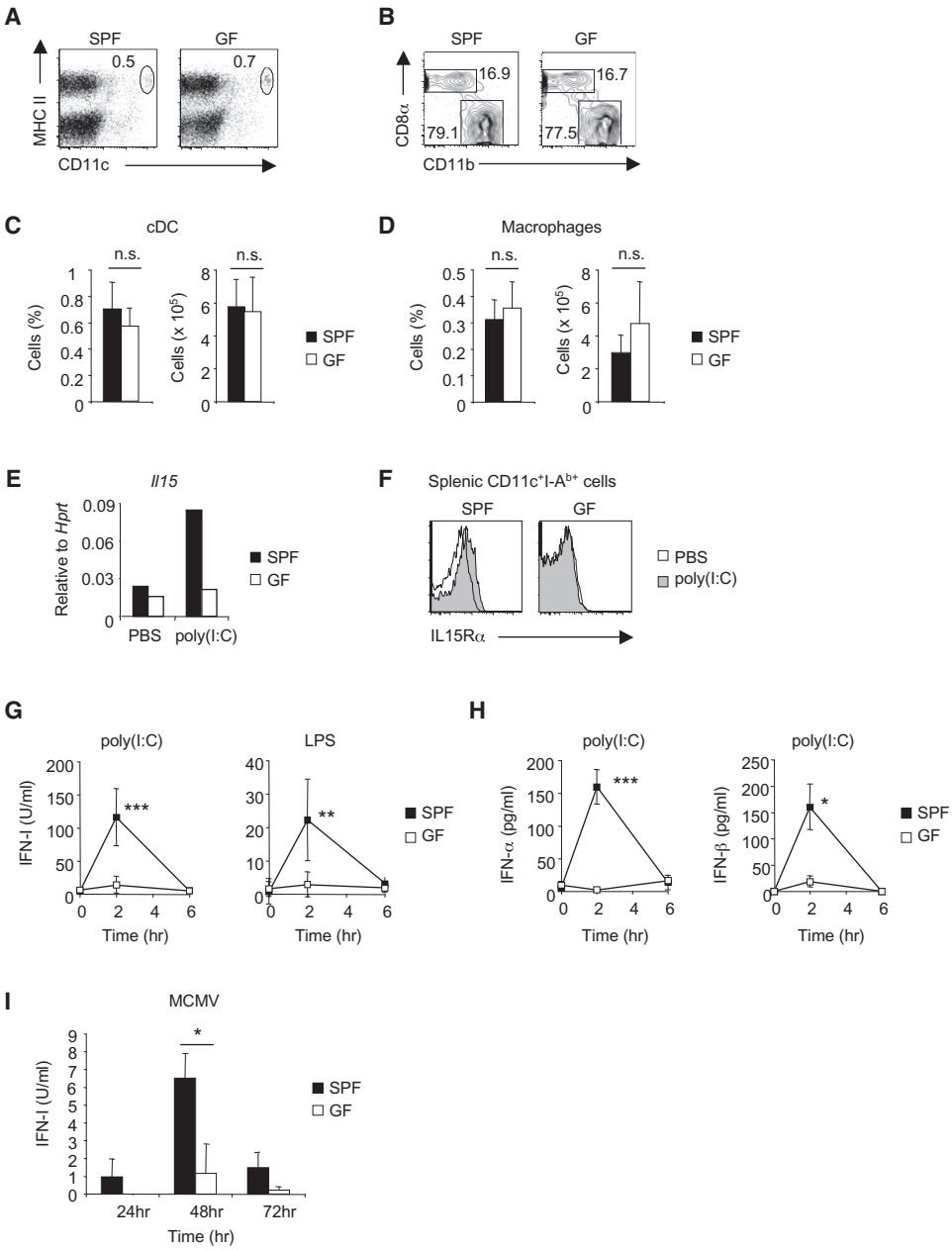

Figure 4. Mononuclear Phagocytes of Germ-free Mice Do Not Produce IFN-I

(A and 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 $CD11b^+CD8\alpha^-$ or $CD11b^-CD8\alpha^+$ DCs (B).

(C) Relative and absolute splenic DC numbers (mean ± SD, n ≥ 5).

(D) Relative and absolute numbers (mean ± SD, n ≥ 5) of splenic $CD11b^+F4/80^+$ macrophages.

(E and F) Groups of mice were injected with poly(I:C), splenic DCs were isolated after 12 hr, and *Il15* expression was analyzed with quantitative (q) RT-PCR (E). IL-15Rα expression by unstimulated (open) and poly(I:C)-stimulated (gray) cDCs (F).

(G) Groups of mice were stimulated with the indicated TLR ligands and serum IFN-I levels were determined at the indicated time points via the VSV bioassay. Data represent mean (±SD, n ≥ 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 (±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 hr via the VSV bioassay. Data represent mean (±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.

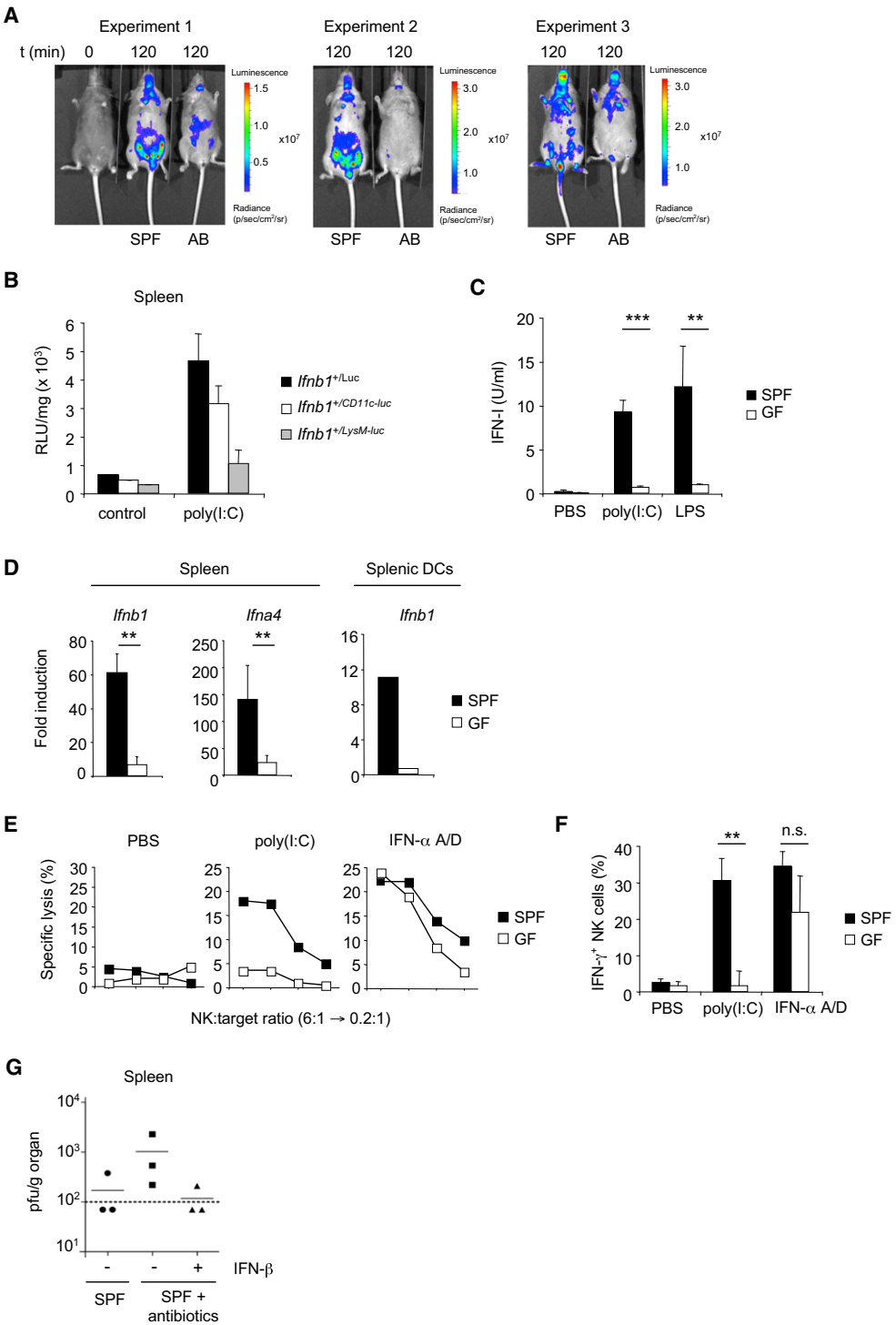

Figure 5. Systemic Failure of Pathogen-Induced IFN-I Production in Germ-free Mice

(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 with a bioluminescence camera. Representative pairs of mice from three individual experiments are shown.

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

(C) Purified DCs from SPF and GF mice were cultured in vitro in the presence of poly(I:C) (5 μg/ml) or LPS (100 ng/ml) for 16 hr. IFN-I levels in the tissue culture supernatants were determined by VSV bioassay. Data represent mean (±SD, n = 5).

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

neutrophils (*Ifnb1*$^{+/LysM\text{-}luc}$ mice). Injection of poly(I:C) into *Ifnb1*$^{+/Luc}$, *Ifnb1*$^{+/CD11c\text{-}luc}$, or *Ifnb1*$^{+/LysM\text{-}luc}$ mice demonstrated that splenic CD11c$^+$ DCs are a substantial source of IFN-β (~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 after 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$^+$ DCs from SPF and germ-free mice and stimulated them in vitro to diminish effects extrinsic to DCs. Intriguingly, highly purified DCs from germ-free mice did not produce IFN-I in response to microbial stimulation whereas DCs 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 DCs after 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 demonstrate that defective NK cell responses in germ-free mice are due to a systemic failure of mononuclear phagocytes such as DCs to produce IFN-I in response to microbial stimulation.

Our data indicate 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 (Figure S4E) after infection. We therefore tested whether continuous immune activation through a persisting virus could normalize IFN-I production. Although 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 microbiota-dependent gene expression programs, we analyzed expression of additional microbe-inducible genes by mononuclear phagocytes from germ-free mice. In contrast to IFN-I gene expression, LPS-induced upregulation 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 after microbial stimulation of DCs from germ-free mice (Figures 6C–6F). Similar to IFN-I, this block is transcriptional as shown by the fact that 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 after 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 proinflammatory 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 DCs 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-κB, which, upon activation, translocate to the nucleus and induce transcription (Kawai and Akira, 2006). Both IRF3 and NF-κB are required for optimal induction of IFN-I expression (Smale, 2010; Tamura et al., 2008). The amount of phosphorylated IRF3 (Figures 7A and S6C) and of IκBα (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), whereas 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-κB p65, IRF3,

(E and F) Groups of mice were injected with poly(I:C), human IFN-α A/D, or PBS (control). Cytotoxicity (E) and IFN-γ production (F) of splenic NK cells against YAC-1 targets 16 hr poststimulation. Data represent mean (±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 hr 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 ≤ 0.01, ***p ≤ 0.001, n.s., not significant. See also Figure S4.

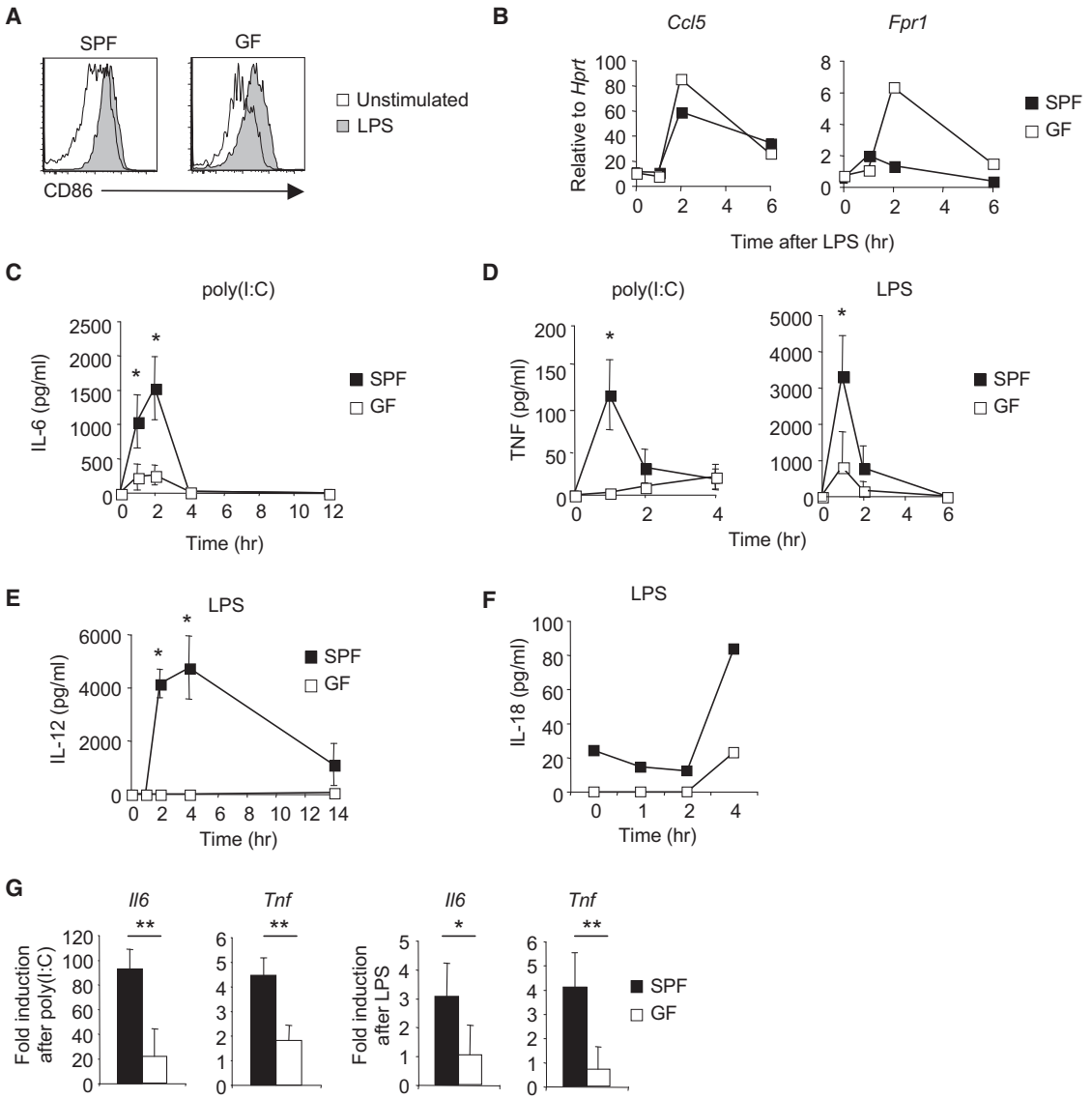

Figure 6. Selective Expression of Pathogen-Inducible Genes in Germ-free Mice

(A) Groups of mice were injected with LPS (gray) or left unstimulated (open). After 3 hr, CD86 expression by CD11c^{hi}MHCII^{hi} cells was determined by 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 (±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 hr (*Tnf*) or 2 hr (*Il6*) later. *Il6* and *Tnf* expression were analyzed by qRT-PCR. Data represent fold induction (mean ± SD, n = 5) of gene expression after the TLR stimulus.

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

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 DCs from SPF mice led to rapid recruitment of NF-κB 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 DCs (Figures 7D and 7E). Importantly, chromatin binding of NF-κB 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. Consistent with previous data (Ramirez-Carrozzi et al., 2009), analysis of DCs 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 DCs 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 poising the expression of proinflammatory 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 DCs from germ-free mice with inhibitors of histone deacetylases (HDACi) to enhance acetylation of histone proteins. If histone acetylation is low in DCs of germ-free mice, HDACi treatment may restore responsiveness to microbial stimulation. Interestingly, HDACi treatment of DCs 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 nonhematopoietic cells repressing *Tnf* expression (Kruys et al., 1993). We tested whether removal of methyl groups from chromatin by treating DCs from germ-free mice with 5-azacytidine would restore responsiveness to microbial stimulation. DCs 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 DCs from germ-free mice, indicating that differences in DNA methylation are unlikely to fully explain why DCs 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 DCs 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 demonstrate 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 of genes 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 DCs to the mucosa-draining lymph nodes, resulting in reduced priming of antiviral 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 nonmucosal lymphoid organs.

It was unexpected that the commensal microbiota had such a profound impact on the readiness of mononuclear phagocytes residing in nonmucosal lymphoid organs. There is a paucity of data concerning the cytokine responses of nonmucosal mononuclear phagocytes from germ-free mice. Collective evidence from early analyses reveals that differentiation and maturation of macrophages (Bauer et al., 1963; Mørland et al., 1979; Shelton et al., 1970) and DCs (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; Mørland et al., 1979). A recent report has provided a possible framework for how tissue-resident immune cells in nonmucosal 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 probably 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 nonmucosal sites (Clarke et al., 2010). Although this study provides a rationale for how microbiota signals may be communicated to sterile peripheral lymphoid organs, NOD1-dependent sensing of commensal microbiota is probably 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 nonmucosal lymphoid organs.

The experiments with antibiotic-treated and germ-free mice recolonized 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 DCs or their circulating progenitors. Previous parabiosis experiments indicated that splenic DCs 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

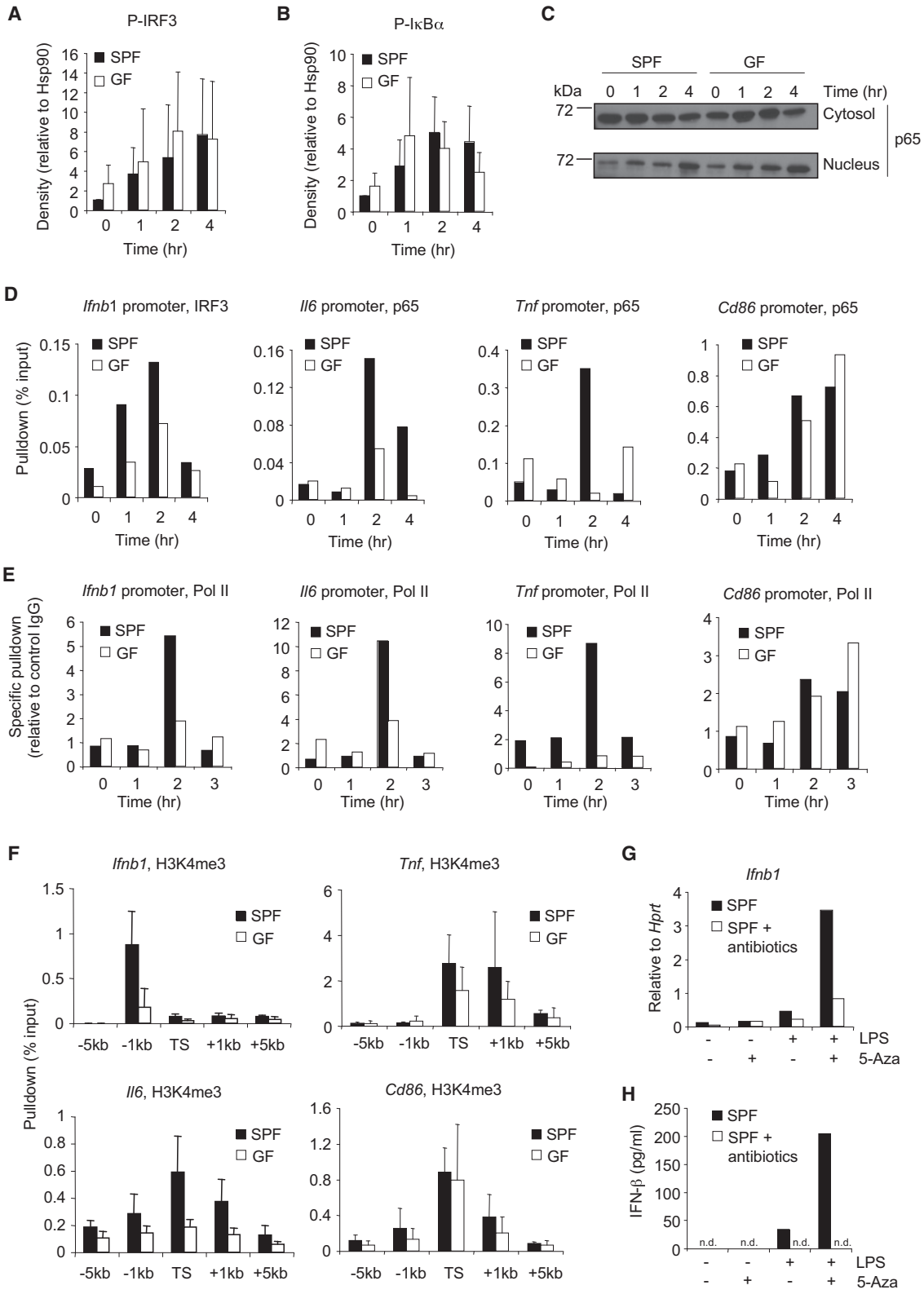

Figure 7. Increased Barrier for NF-κB and IRF3 Chromatin Binding in Germ-free DCs

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

respond to microbial stimulation because highly purified mononuclear phagocytes remained unresponsive when stimulated in vitro. Collectively, our data provide a unique 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 nonmucosal 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-κB 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 microbiota. 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-κB 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 DCs, as previously indicated (Hargreaves et al., 2009; Ramirez-Carrozzi et al., 2009). 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 poising 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 DCs from SPF mice led to enhanced production of cytokines, it did not restore cytokine production in germ-free DCs. These data indicate that DNA hypermethylation is unlikely to be the underlying epigenetic signature repressing cytokine production by DCs 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 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, probably at the level of chromatin. This leads to poising of inflammatory gene expression, thereby calibrating mononuclear phagocytes at sterile, nonmucosal 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).

(C) DCs 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-κB p65 determined by immunoblot analysis.
(D) DCs were purified from SPF and GF mice and stimulated with LPS in vitro. IRF3 and NF-κB 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 pull down relative to a DNA input sample. Pull down with a control antibody was significantly lower or absent.
(E) DCs were purified from SPF and GF mice and stimulated with LPS in vitro. RNA Pol II ChIP was performed to determine specific pull down of RNA Pol II at the transcriptional start sites of the indicated genes. Results represent specific pull down relative to a DNA input sample normalized to pull down with a control antibody.
(F) DCs were purified from SPF and GF mice. H3K4me3 ChIP was performed and specific pull down was determined around the transcriptional start (TS) sites of the indicated genes. Results represent specific pull down relative to a DNA input sample. Pull down with a control antibody was significantly lower or absent.
(G and H) Groups of mice were treated in vivo with 5-azacytidine (5-Aza) or left untreated. Subsequently, splenic DCs were isolated and stimulated ex vivo in the presence of 0.1 μg/ml LPS for 4 hr. *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.

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 hr ^{51}Cr 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 Interferon-source) 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 with 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) via Lysing Matrix A on a FastPrep-24 (MP Biomedicals). Luciferase activity was measured in a Sirius Tube Luminometer (Berthold Technologies) by the single Luciferase Assay System (Promega).

Chromatin Immunoprecipitation

Splenic DCs were isolated with CD11c beads (Miltenyi). DCs (3 × 10^6) were stimulated in vitro with 0.1 μg/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 previsouly 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.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.05.020.

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