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IL-15 *cis* Presentation Is Required for Optimal NK Cell Activation in Lipopolysaccharide-Mediated Inflammatory Conditions

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SUMMARY

Natural killer (NK) cells have antitumor, antiviral, and antibacterial functions, and efforts are being made to manipulate them in immunotherapeutic approaches. However, their activation mechanisms remain poorly defined, particularly during bacterial infections. Here, we show that upon lipopolysaccharide or *E. coli* exposure, dendritic cells (DCs) produce three cytokines—interleukin 2 (IL-2), IL-18, and interferon β (IFN-β)—necessary and sufficient for NK cell activation. IFN-β enhances NK cell activation by inducing IL-15 and IL-15 receptor α not only in DCs but, surprisingly, also in NK cells. This process allows the transfer of IL-15 on NK cell surface and its *cis* presentation. *cis*-presented NK cell-derived and *trans*-presented DC-derived IL-15 contribute equally to optimal NK cell activation.

INTRODUCTION

Natural killer (NK) cells are lymphocytes of innate immunity equipped only with germline-encoded receptors not undergoing somatic rearrangement. Most of them are specifically designed to monitor major histocompatibility complex (MHC) and MHC-like molecules on host cells, promptly detecting stressful alterations, usually due to viral infection or transformation (Horowitz et al., 2012). Upon recognition of abnormal cells, NK cells release the content of cytolytic granules, leading to the death of target cells.

Cytotoxicity in antiviral and antitumor responses is the traditionally acknowledged contribution of NK cells to immunity, but it is by no means the only one. NK cells are also powerful producers of proinflammatory cytokines, particularly interferon γ (IFN-γ), of which NK cells are the earliest source in several types of infections (Horowitz et al., 2012; Martín-Fontecha et al., 2004). IFN-γ potently activates macrophages and favors Th1 lineage commitment of $CD4^+$ T cells. As such, it comes as no surprise that early IFN-γ release by NK cells is crucial to control a variety of infections by intracellular (such as *Listeria monocytogenes*) and extracellular (such as *Escherichia coli*) bacteria while adaptive immunity is being primed (Dunn and North, 1991; Ferlazzo et al., 2003; Lapaque et al., 2009; Newman and Riley, 2007; Pontiroli et al., 2012). This raises the question of how NK cells are alerted by the presence of bacteria, as NK cells are classically thought not to bear any receptor-recognizing bacterial products. In principle, detection of intracellular bacteria by NK cells might still be possible through their well-known patrolling mechanism, which seeks stress-related alterations in host cells. However, recognition of extracellular bacteria is harder to envision. Recently, two important lines of research have uncovered important aspects of NK cell biology that might shed light on this topic. First, both mouse and human NK cells express Toll-like receptors (TLRs) (Lauzon et al., 2007), which recognize molecular patterns associated to pathogens (PAMPs) (Medzhitov et al., 1997), and second, accessory cells, such as dendritic cells (DCs), are able to sense the presence of pathogens and, in turn, activate NK cells, thus virtually expanding NK cell recognition capabilities (Fernandez et al., 1999; Newman and Riley, 2007).

The contribution of autonomous pathogen recognition by NK cells through TLRs is debated, with some works showing enhanced effector functions (Lauzon et al., 2006; Pisegna et al., 2004; Schmidt et al., 2004; Sivori et al., 2004) upon stimulation of NK cell TLRs, whereas others report no impact (Gorski et al., 2006; McCartney et al., 2009; Newman and Riley, 2007). To reconcile those discordant data, some authors proposed that the role of NK cell TLRs might become apparent only in synergism with accessory cell-derived cytokines (Lauzon et al., 2007; Sivori et al., 2004). By contrast, the role of accessory cells in NK cell activation is better appreciated. In 1999, a pioneering work by Fernandez et al. (1999) formally demonstrated that DCs are able to activate NK cells via the joint contribution of both

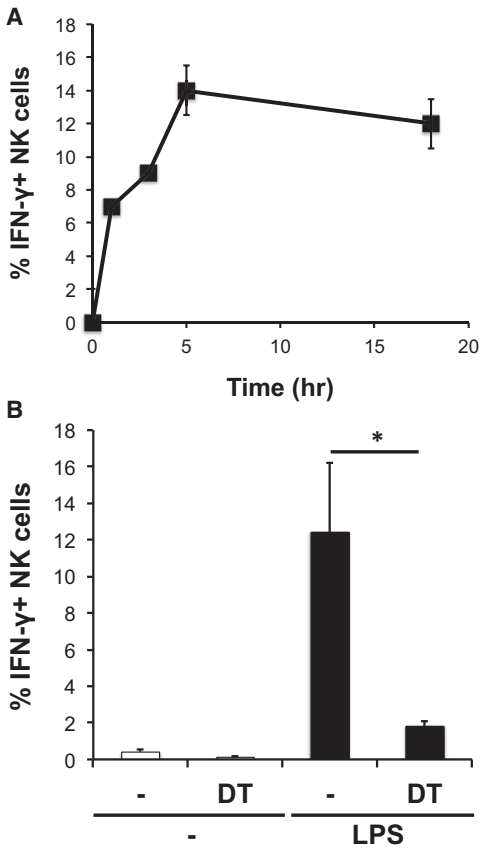

Figure 1. DCs Are Required for NK Cell Activation

(A) Activated (IFN-γ-positive) NK cells, defined as CD49b$^+$ CD3$^-$, analyzed by intracellular staining at the indicated time points in the draining lymph node after LPS s.c. injection.

(B) Activated (IFN-γ-positive) NK cells in CD11c.DOG mice before (-) and 5 hr after LPS injection. Where indicated, mice were pretreated for 4 hr with DT to eliminate DCs.

$n \geq 3$. Error bars depict SEM. Statistical significance was determined with a two-tail t test. *, $p < 0.05$.

See also Figure S1.

contact-dependent and contact-independent mechanisms. This observation has been repeatedly confirmed in a variety of experimental settings. Yet there is little agreement about the mechanism through which DCs activate NK cells (Ferlazzo et al., 2002; Newman and Riley, 2007).

Moreover, confusion can arise from the finding that accessory cells may not only activate NK cells—resulting in immediate effects, such as cytokine production at the site of interaction—but also "prime" them by lowering their threshold of activation. Priming would favor subsequent responses at the site of restimulation/target recognition, likely differing from the priming site (Chaix et al., 2008; Lucas et al., 2007). Accordingly, interactions between NK cells and accessory cells may occur in peripheral tissues—where NK cell activation would result in IFN-γ release, able to activate macrophages—and/or in secondary lymphoid organs—where NK cell-derived IFN-γ would be pivotal for Th1 polarization (Boehm et al., 1997; Martín-Fontecha et al., 2004; Moretta et al., 2006; Zanoni et al., 2005). The molecular signals driving either activation or priming may only partially overlap, requiring investigators to distinguish between these two alternatives.

In this work, we aim at deciphering the mechanisms underlying NK cell activation during Gram-negative bacterial infections. Activation was induced with *E. coli* and its major PAMP, the lipopolysaccharide (LPS), for which an extensive body of knowledge and a broad array of genetic tools to dissect its immune response are available (Zanoni et al., 2011). TLR ligands have been widely used in recent studies of NK cell activation (Beuneu et al., 2009; Ebihara et al., 2010; Lucas et al., 2007; McCartney et al., 2009; Miyake et al., 2009; Newman and Riley, 2007; Zanoni et al., 2005, 2012), endowing this choice with the additional value of immediacy when linking this work to existing literature.

Whereas the main emphasis was on the identification of molecular events leading to IFN-γ secretion—the chief antibacterial weapon of NK cells (Boehm et al., 1997; Granucci et al., 2004)—we did not neglect to compare whether similar requirements applied to cytotoxic functions as well.

Here, we provide a comprehensive picture of the molecular and cellular events underlying NK cell activation in vitro and in vivo in response to LPS and Gram-negative bacteria. The contribution of key cytokines in NK cell activation, such as interleukin 15 (IL-15), may be revisited in light of our findings. Understanding the molecular mechanisms of NK cell activation has not only important implications in fundamental biology but also a marked translational impact, given the increasing interest in exploiting and manipulating NK cell functions in immunotherapy (Murphy et al., 2012).

RESULTS

DCs Are Essential Drivers of NK Cell Activation under LPS-Induced Inflammatory Conditions

A productive DC-NK cell crosstalk may result in NK cell priming, activation, or both. To assess whether NK cells were successfully primed by accessory cells, investigators have often restimulated NK cells using target cells, antibodies against activating receptors, or PMA/ionomycin before measuring IFN-γ release (Lucas et al., 2007; Martín-Fontecha et al., 2004). This strategy captures the contribution of accessory cells in lowering the threshold of NK cell activation, but it does not give insights into direct NK cell activation right after (or even during) interaction with DCs.

We investigated ex vivo NK cell activation in the absence of restimulation. LPS was injected in the anterior footpad to induce inflammation, and the percentage of IFN-γ-producing NK cells in the draining lymph node was measured at different time points. IFN-γ-positive NK cells could be identified with a maximal peak of 15% at 5 hr after LPS injection (Figure 1A). The same experiment was then repeated using CD11c.DOG mice (Hochweller et al., 2008), in which CD11c^{high} cells (DCs) express the diphtheria toxin (DT) receptor (DTR) and can be depleted upon DT injection. In the absence of DCs, a profound drop in the percentage of activated NK cells was observed (Figures 1B and S1).

These results argued in favor of a pivotal role of DCs in direct NK cell activation (with immediate Th1-polarizing IFN-γ release [Martín-Fontecha et al., 2004]), in addition to the well-characterized NK cell priming (with IFN-γ released in the periphery after target encounter). Moreover, the requirement of DCs and the kinetic of NK cell activation suggest that DC-NK cell interactions take place in vivo early after inflammation onset.

DC-Derived IL-2, IL-18, and IFN-β Are Necessary and Sufficient to Induce IFN-γ Release by NK Cells in Response to LPS In Vitro

Having confirmed that DCs activate NK cells, we next set to characterize the underlying molecular framework, starting with in vitro studies.

Upon stimulation with LPS, bone marrow (BM)-derived DCs acquired the ability to directly elicit IFN-γ production by NK cells. TLR4 and both the adaptors MyD88 and Toll-IL-1 receptor (TIR)-containing adaptor molecule 1 (TICAM1) (also known as TIR domain-containing adaptor-inducing IFN-β, TRIF) were required for this process (Figure 2A). Interestingly, NK cells could undergo full activation in the absence of TLR4 and TICAM1 but not MyD88 (Figure 2B). Thus, despite that intracellular TLRs might play a role in promoting NK cell activation directly (Sivori et al., 2004), the plasma membrane-bound TLR4 is dispensable.

The requirement for MyD88 but not TLR4 on NK cells (Figure 2B) for IFN-γ release in LPS-stimulated DC-NK cell cocultures suggested the involvement of IL-1 family receptors, which signal through MyD88 (Dinarello, 2009). Among others, IL-1α, IL-1β, and IL-18 are members of the IL-1 family. IL-1α and IL-1β share the type I IL-1 receptor (IL-1R), whereas IL-18 is recognized by IL-18R. We inhibited IL-1 activity with neutralizing antibodies to IL-1R or with the recombinant IL-1R antagonist (IL-1RA)—another member of the IL-1 family (Dinarello, 2009)—or with an anti-IL-1β-blocking antibody. The first two treatments neutralize both IL-1α and IL-1β, whereas the latter spares IL-1α. We found that in no case were NK cell-derived IFN-γ levels affected, ruling out a role for IL-1α and IL-1β in DC-mediated NK cell activation (Figure 2C). By contrast, blocking IL-18 with either recombinant IL-18 binding protein (IL-18BP) or neutralizing antibodies against the cytokine itself or its receptor effectively dampened IFN-γ release by NK cells (Figure 2C). These results were confirmed by using NK cells purified from mice deficient for either IL-18R or IL-1R. *Il18r1*$^{-/-}$ (but not *Il1r1*$^{-/-}$) NK cells showed a marked reduction of IFN-γ release (Figure 2D). Similarly, IL-18-deficient BM-derived DCs (BM-DCs) could not fully activate NK cells, and the addition of recombinant IL-18 at the dose of 120 pg/ml completely restored IFN-γ production (Figure 2E). In summary, LPS-matured DCs elicited efficient IFN-γ release from NK cells through the IL-18R-MyD88 axis.

The requirement of TICAM1 on DCs to obtain IFN-γ release by NK cells (Figure 2A) could imply a role for IFN-β, a cytokine totally controlled by the LPS-activated TICAM1 pathway (Yamamoto et al., 2002; Zanoni et al., 2011). To test this hypothesis, we took advantage of IFN-β-deficient DCs. As shown in Figure 2F, these mutant cells were far less capable of stimulating IFN-γ production by NK cells than the wild-type (WT) counterpart. This phenotype was reversed by the addition of recombinant IFN-β (rIFN-β) (Figure 2F), demonstrating that the impairment of NK cell activation was truly due to the absence of IFN-β.

Concerning the requirement for MyD88 on DCs, we focused our analysis on three cytokines known to be produced in a MyD88-dependent way following LPS exposure and previously described to contribute to IFN-γ release by NK cells, namely IL-12p70, tumor necrosis factor α (TNF-α), and IL-2. IL-12p70 has been classically linked to NK cell activation and IFN-γ release in humans in response to viral and bacterial stimuli (Ferlazzo et al., 2002; Fink et al., 2007; Muntasell et al., 2010; Newman and Riley, 2007; Romo et al., 2011) and in mice during viral infections (French and Yokoyama, 2003; Nguyen et al., 2000; Orange and Biron, 1996). To investigate whether this was the case in our system, we blocked IL-12 in DC-NK cell cocultures. As shown in Figure 2G, IL-12 neutralization did not dampen IFN-γ production. Similarly, we ruled out a role for TNF-α (data not shown). In contrast, we confirmed the contribution of IL-2 (Figure S2), as already shown by our group (Granucci et al., 2004). Finally, we also excluded a role for CD70 stimulation (Figure S2), previously suggested to favor the release of IFN-γ by NK cells (Newman and Riley, 2007; Takeda et al., 2000).

In summary, we could establish that DC-derived IL-2, IL-18, and IFN-β were essential signals for NK cell activation. To test whether they were not only necessary but also sufficient to trigger NK cell activation, we used recombinant IL-2, IL-18, and IFN-β to stimulate NK cells. Each cytokine was added at the concentration restoring full IFN-γ production when NK cells were cultured with the relevant cytokine-deficient DCs (Figures 2E and 2F; data not shown), thereby avoiding over- or understimulation of NK cells. We found that the simultaneous stimulation of NK cells by IL-2, IL-18, and IFN-β elicited IFN-γ release, whereas the lack of any of the three cytokines resulted in a totally ineffective stimulation (Figure 2H).

In conclusion, LPS-activated DCs were able to directly activate NK cells in vitro by secreting three necessary and sufficient signals: IL-2, IL-18, and IFN-β.

IL-18 and IFN-β Are Required for NK Cell Activation In Vivo

Having established the in vitro relevance of IL-2, IL-18, and IFN-β in NK cell activation under LPS-mediated inflammatory conditions, we analyzed whether IL-18 and IFN-β were required in vivo for NK cell activation following exposure to whole Gram-negative bacteria. In a previous work, we have already demonstrated the essential role of IL-2 in NK cell activation during *E. coli* infections (Granucci et al., 2004). In agreement with our in vitro data, 5 hr after intravenous (i.v.) injection of *E. coli,* the fraction of IFN-γ^{+} NK cells in the spleen was strongly diminished in IL-18- and IFN-β-deficient mice (Figure 3A). By contrast, the absence of IL-12p35 had a negligible effect on NK cell activation (Figure 3A), again confirming our in vitro data (Figure 2G). This observation was somehow in contrast with the established role of IL-12 in NK cell activation during viral infections in mice (French and Yokoyama, 2003; Nguyen et al., 2000; Orange and Biron, 1996) and in response to viral and bacterial stimuli in humans (Ferlazzo et al., 2003, 2004; Fink et al., 2007; Muntasell et al., 2010; Romo et al., 2011). Because the upregulation of *Il12b* mRNA in DCs in response to *E. coli* is relatively late, taking more than 4 hr

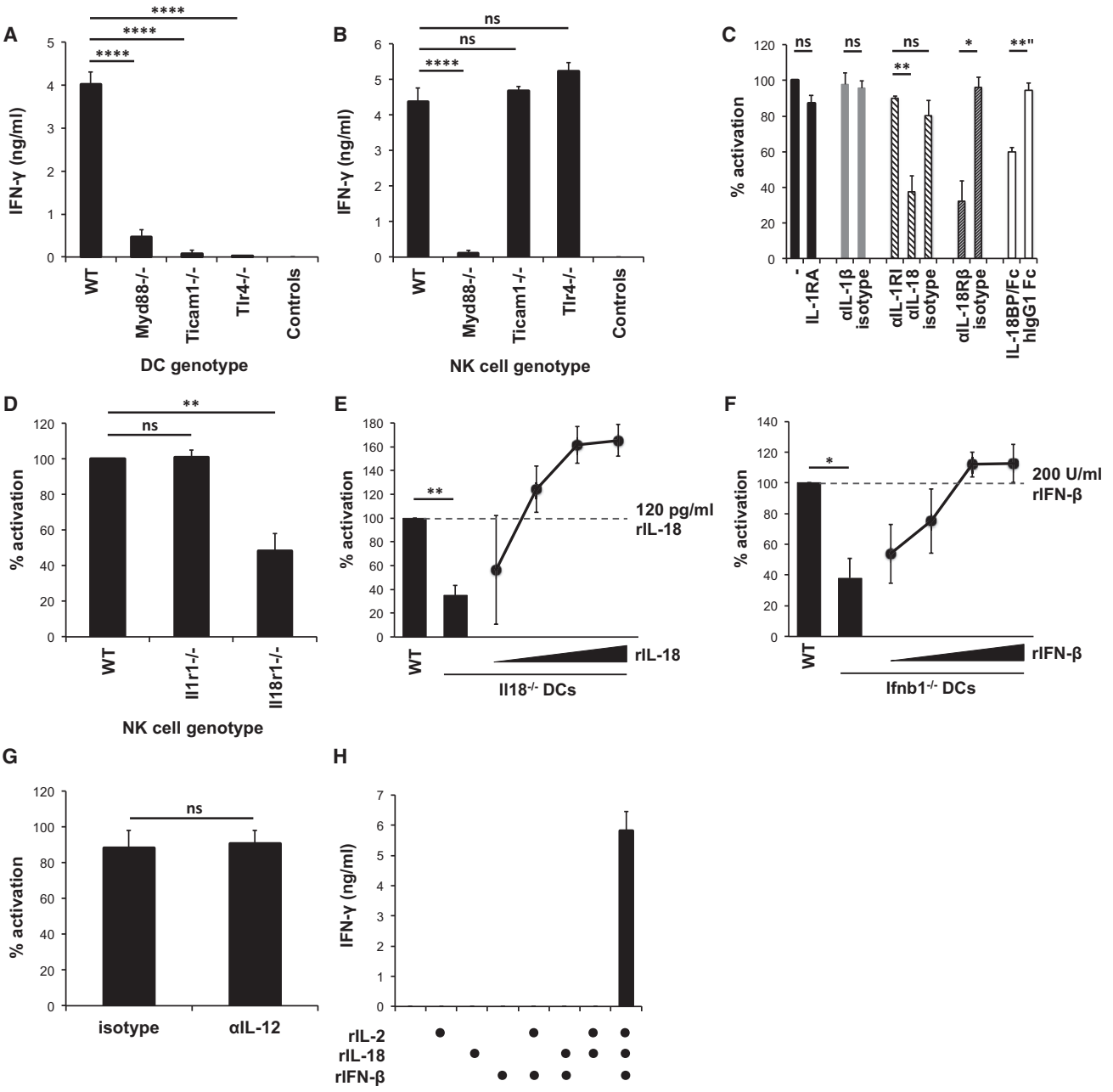

Figure 2. IL-18, IL-2, and IFN-β Are Necessary and Sufficient to Induce IFN-γ Release in NK Cells Activated by LPS-Stimulated BM-DCs

After coculturing BM-DCs and NK cells for 18 hr, NK cell-derived IFN-γ secreted in the supernatant was measured by ELISA.

(A) LPS-stimulated BM-DCs of the indicated genotype were cocultured with WT NK cells.

(B) LPS-stimulated WT BM-DCs were cocultured with NK cells of the indicated genotype. (A and B) Controls: DC + NK; DC + LPS; NK + LPS; DC; NK.

(C) NK cells and BM-DCs were cocultured in the presence of the indicated neutralizing Abs (or isotype controls) or recombinant proteins.

(D) NK cells of the indicated genotype were cocultured with LPS-stimulated WT BM-DCs.

(E and F) LPS-stimulated BM-DCs of the indicated genotype were cocultured with WT NK cells. Where indicated, increasing doses of rIL-18 or rIFN-β were added to the cocultures.

(G) WT BM-DCs and WT NK cells were cocultured in the presence of LPS and the indicated neutralizing Ab (or isotype control). (D–G) Results are shown as percentage of IFN-γ release relative to WT NK cells activated by LPS-stimulated WT BM-DCs.

(H) NK cells were cultured without BM-DCs in the presence or absence of rIL-2 (1 ng/ml), rIL-18 (120 pg/ml), and rIFN-β (200 U/ml).

(A and B) $n \geq 4$. (C–H) $n \geq 3$. Statistical significance was determined with ANOVA followed by Dunnett's multiple comparison test (A and B, white-and-black stripes in [C] and [D]), a two-tailed one-sample t test (solid black in [C], [E], and [F]) or a two-tailed two-sample t test (all others in [C] and [G]). Error bars depict SEM.

*, $p < 0.05$; **, $p < 0.01$; ***,$p < 0.001$; ****, $p < 0.0001$; ns, not significant.

See also Figure S2.

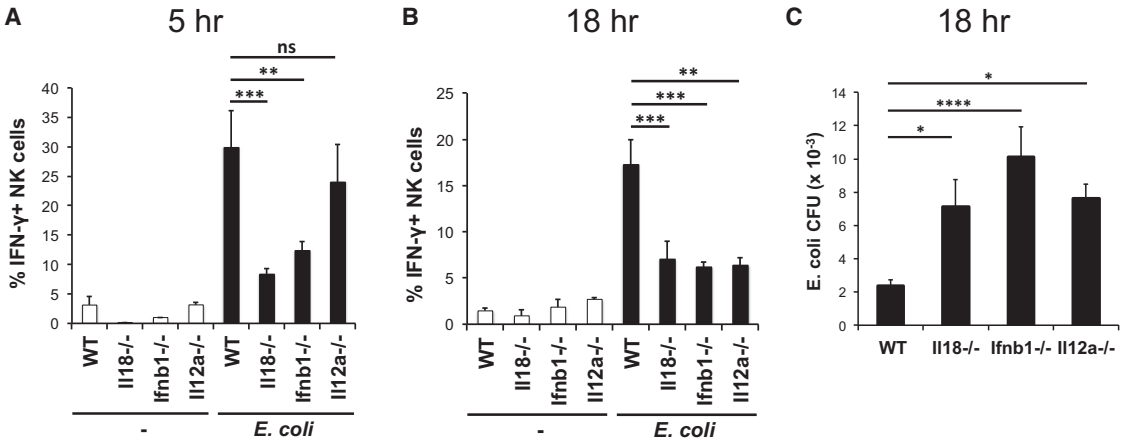

Figure 3. Role of IL-18, IFN-β, and IL-12 in Antibacterial Immunity

(A–C) Mice were challenged i.v. with *E. coli*. NK cell activation at 5 (A) and 18 (B) hr was determined in the indicated knockout mice. Residual bacterial burden in the spleen was measured at 18 hr (C). (A and B) n = 4–8 mice per group from two independent experiments. (C) n = 6–27 mice per group from four independent experiments. Statistical significance was determined with ANOVA followed by Dunnett's multiple comparison test. Error bars depict SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant. See also Figure S3.

(Granucci et al., 2001), we hypothesized that IL-12, although dispensable at early time points, could rather play a role in maintaining NK cell activation at late time points. Therefore, we measured the effect of IL-12p35 deficiency on IFN-γ secretion by NK cells 18 hr after bacterial injection. As shown in Figure 3B, a significant decrease in the fraction of IFN-γ^+ NK cells could be observed in IL-12p35-deficient animals. The contribution of IL-18 and IFN-β was detectable also at late time points (Figure 3B). Consistent with the roles of IL-18, IFN-β, and IL-12 in NK cell activation, *E. coli* clearance in the spleen was significantly reduced in IL-18-, IFN-β-, and IL-12p35-deficient mice 18 hr after bacterial challenge (Figure 3C). These data indicate that, in addition to IL-2 (Granucci et al., 2004), IL-18 and IFN-β are required in vivo to initiate the process of NK cell activation during Gram-negative bacterial infections and that IL-12 is later required to maintain the size of the activated NK cell population.

DC-Derived IL-2, IL-18, and IFN-β Are Required for Full IFN-γ Release by NK Cells In Vivo

We next aimed to establish whether DCs had a critical role in vivo in secreting the cytokines IL-2, IL-18, and IFN-β, which initiate the process of NK cell activation. Because the in vivo responses to *E. coli* and LPS at early time points were almost completely superimposable (Figures S3 and 3A)—confirming previous observations that DC- and macrophage-driven responses to *E. coli* were mediated by TLR4 (Poltorak et al., 1998; Takeuchi et al., 1999; Zanoni et al., 2011)—we performed the following experiments using LPS as a stimulus.

We conceived a mixed BM chimera model to largely restrict a candidate cytokine deficiency to DCs (Figure 4A). To this aim, we took again advantage of CD11c.DOG mice (Hochweller et al., 2008). For labeling, these animals were crossed with ubiquitin.GFP transgenic mice, giving rise to DOG-GFP double transgenic (dTg) animals. In our mixed BM chimeras, 80% of donor cells came from DOG-GFPdTg mice and 20% came from mice deficient for a selected cytokine—IL-2, IL-18, or IFN-β—or TLR4, as a negative control (Figure 4A). Upon injection of DT, only DOG-GFPdTg-derived CD11c^{high} cells were ablated. Therefore, precursors from the DT-insensitive but cytokine-deficient fraction were allowed to replenish the DC compartment over time. When DT was injected for long enough to allow full replenishment of the DC niche (1 week in our studies), the large majority of DCs were GFP-negative and, consequently, deficient for the cytokine under investigation (Figure S4A). By contrast, the NK cell compartment segregated according to the initial ratio of reconstitution, namely 80% DOG-GFPdTg and 20% knockout (KO) for a given cytokine, even after DT treatment (Figure S4B). Therefore, most radiation-sensitive non-DC cells, including NK cells, did not bear any genetic deficiency, regardless if DT was injected or not, and the minor cytokine-KO fraction was readily distinguishable by GFP expression (Figure 4A). Importantly, the DT treatment did not alter the percentage of activated NK cells after LPS administration (Figure S4C). Moreover, cytokine-deficient NK cells could be activated as efficiently as WT NK cells (Figure S4D), indicating that the genetic mutations under investigation did not intrinsically affect NK cell functionality.

LPS was injected in mixed BM chimeras after 1 week of daily DT treatment, and NK cell activation in the spleen was assessed 5 hr later (Figure 4A). In line with our expectations, the IFN-γ^+ fraction of NK cells was reduced when DCs lacked any of the three cytokines (Figure 4B), confirming the requirement for DC-derived IL-2, IL-18, and IFN-β in NK cell activation in vivo upon LPS stimulation.

IL-15 Is Required for NK Cell Activation in Response to IFN-β and Can Be Produced by Both DCs and NK Cells

Recently, several investigators have reported a role of the *trans*-presented IL-15 in DC-mediated NK cell activation (Koka et al., 2004; Lucas et al., 2007; Mortier et al., 2008; Newman and Riley, 2007). In this context, type I IFNs would exert their function by eliciting the production of IL-15 from DCs (Lucas et al., 2007; Zhang et al., 1998). To investigate whether this was the

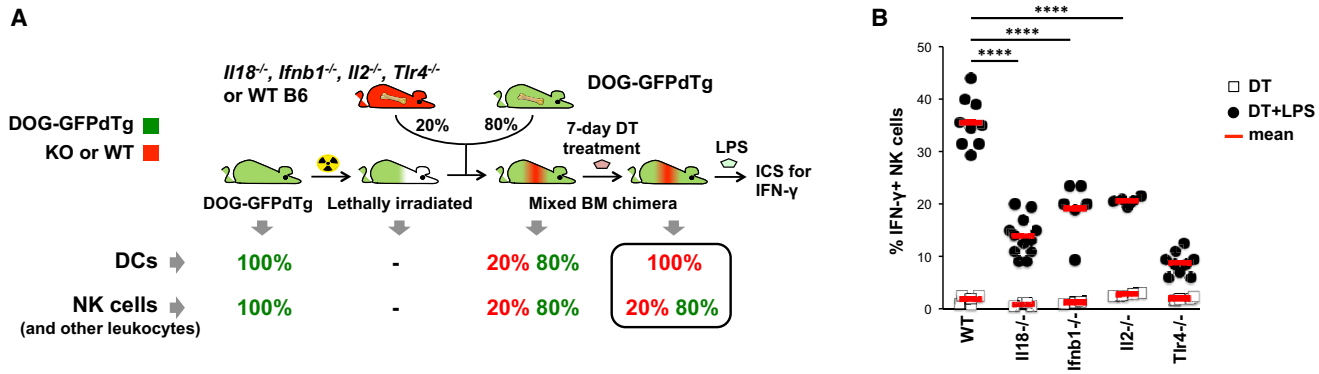

Figure 4. DC-Derived IL-2, IL-18, and IFN-β Are Required for Full NK Cell Activation In Vivo

(A) Experimental design. DOG-GFPdTg mice were lethally irradiated and reconstituted with BM donor cells coming for 80% from DOG-GFP dTg mice and for 20% from mice KO for the indicated genes. After full recovery, mice were treated with DT for 1 week to deplete cytokine-sufficient DCs, allowing KO precursors to replenish the DC niche. As a result, the cytokine deficiency was largely restricted to DCs. One day after the last DT injection, mice were challenged with LPS, and the percentage of activated (IFN-γ^+) NK cells was determined at 5 hr.

(B) Results of the experiment depicted in (A). NK cells were gated as GFP$^+$ CD49b$^+$ CD3$^-$. n = 6–12 mice per group from three independent experiments. Statistical significance was determined with ANOVA followed by Dunnett's multiple comparison test. ****, $p < 0.0001$.

See also Figure S4.

case in our experimental conditions, we tested if the addition of rIL-15 restored IFN-γ secretion by NK cells cocultured with LPS-activated, IFN-β-deficient DCs. Figure 5A shows that this was indeed the case. This observation confirmed the published finding (Lucas et al., 2007) that IFN-β exerts its stimulatory function by inducing IL-15 production.

Surprisingly, however, the ability of IL-15-deficient DCs to activate NK cells was largely preserved (Figure 5B). This could be explained if DCs were not the only source of IL-15. In that case, NK cells should be able to self-produce IL-15 upon IFN-β stimulation. To test this hypothesis, we first measured the ability of NK cells to produce IL-15 in response to IFN-β. For this experiment, NK cells were identified as CD49$^+$ CD3$^-$ CD122$^+$ cells. As shown in Figure 5C, NK cells could indeed produce and expose at the cell surface IL-15 upon stimulation with IFN-β. In agreement with this observation, we also observed the up-regulation of *Il15* mRNA in NK cells exposed to IFN-β in both mouse and man (Figures S5 and S6). Moreover, to corroborate the mechanism of IL-15 production, we analyzed IL-15Rα-deficient NK cells. The prediction of this experiment was that IL-15 would not be detectable at the cell surface, due to its instability when not bound to the α receptor (Mortier et al., 2008). Indeed, IL-15 was not detectable in *Il15ra*$^{-/-}$ NK cells, either at the cell surface or in the cytoplasm (Figure 5C). Conversely, in WT NK cells, IL-15 could be detected intracellularly after IFN-β stimulation and brefeldin A treatment (Figure 5C).

Having established that NK cells were a source of IL-15 as much as DCs, we cocultured DCs and NK cells (CD49b$^+$ CD3$^-$; purity >99%), both isolated from IL-15-deficient animals. Now, under conditions where both DC- and NK cell-derived IL-15 was missing, IFN-γ production by NK cells was totally impaired (Figure 5D). Consistently, when anti-IL-15- or anti-IL-15Rα-blocking antibodies were added to cultures of NK cells stimulated with IL-2, IL-18, and IFN-β, a strong reduction in their ability to secrete IFN-γ was observed (Figure 5E). Our results indicate not only that IL-15 is indeed required for NK cell activation, as previously shown (Lucas et al., 2007), but also that both DCs and NK cells can be the source of this cytokine, at least in vitro.

Next, we tested whether this finding held true in vivo. To this purpose, we generated mixed chimeric mice, in which DCs, NK cells, or both were IL-15-deficient, by adapting the model depicted in Figure 4A. In this variation (Figure 6A), we took advantage of NKp46.DTR mice, in which NK cells are labeled (GFP$^+$) and can be conditionally depleted (DTR$^+$) upon DT treatment (Walzer et al., 2007). To target both DCs and NK cells, NKp46.DTR mice were crossed with CD11c.DOG mice. Recipient mice (any one of DOG-GFPdTg, NKp46.DTR, or DOG-GFPdTg × NKp46.DTR mice) were irradiated and reconstituted with mixed BM cells derived for 20% from IL-15-deficient mice and for 80% from donors of the same genotype as recipient mice. Upon DT injection, DCs, NK cells, or both NK cells and DCs were ablated and their niche was replenished by IL-15-deficient precursors, yielding *Il15*$^{-/-}$ DCs, *Il15*$^{-/-}$ NK cells, or both *Il15*$^{-/-}$ DCs and *Il15*$^{-/-}$ NK cells, respectively (Figures 6A and S7). Chimeric mice were then injected with LPS, and the percentage of IFN-γ-positive NK cells in the spleen was determined. NK cell activation was totally impaired only when both DCs and NK cells were unable to produce IL-15 (Figures 6B and S7). These results confirmed our in vitro observation that IL-15 was required for NK cell activation induced by LPS and that both DCs and NK cells could be sources of IL-15, in response to type I IFNs.

In agreement with our observations, we also found a clear increase in *Il15ra* mRNA expression by both murine and human NK cells after IFN-β exposure (Figures 6C and S6). This is in line with the observation by French et al. (2006) of IL-15Rα upregulation by NK cells during murine cytomegalovirus (MCMV) infections. Moreover, functional outcomes observed using IL-15-deficient DCs and NK cells could be recapitulated by their *Il15ra*$^{-/-}$ counterparts (Figures 6D–6F), as it may be expected from the observation that IL-15 and IL-15Rα are physiologically found in complex (Mortier et al., 2008). Indeed, when IL-15Rα-deficient NK cells were stimulated with IL-2, IL-18, and IFN-β,

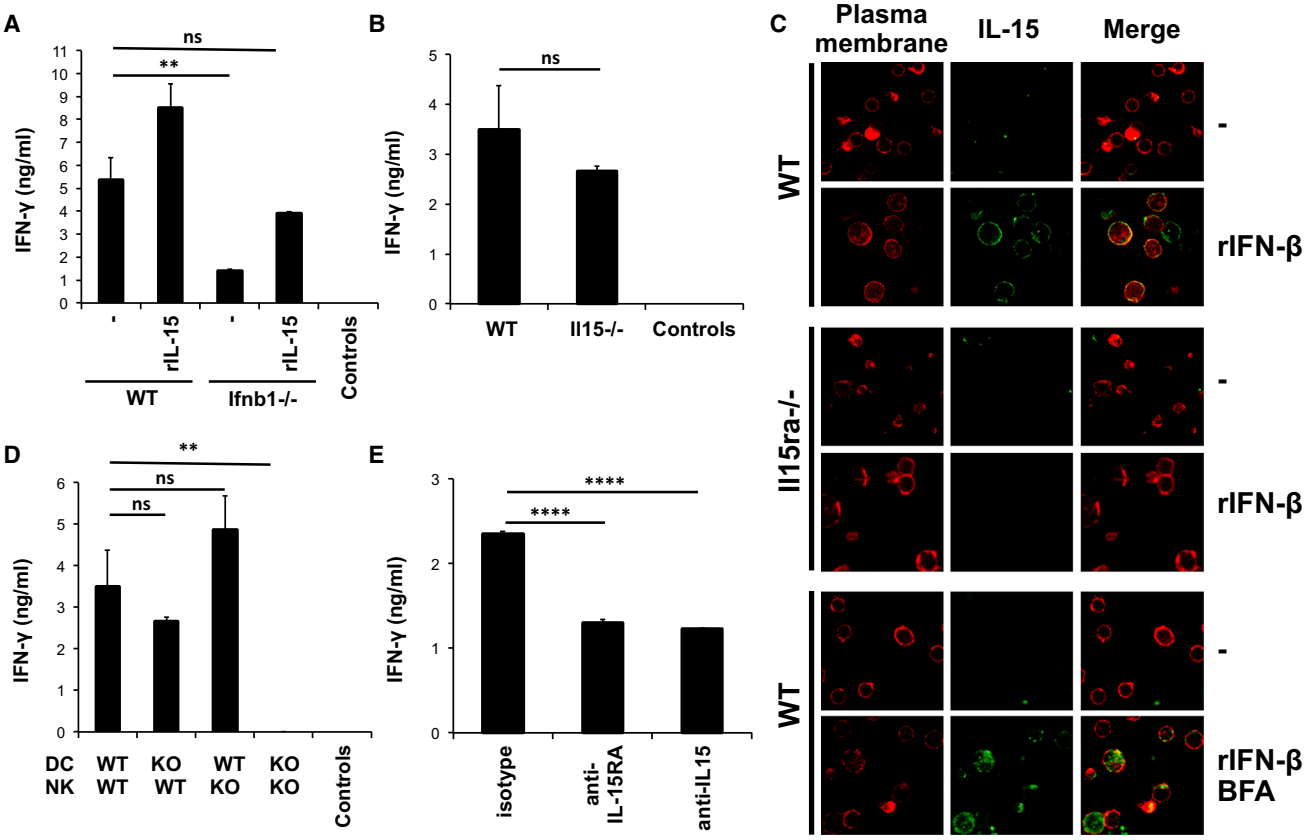

Figure 5. IL-15 Is Required for NK Cell Activation and Can Be Produced by Both DCs and NK Cells In Vitro

(A and B) IFN-γ production by WT NK cells stimulated with LPS-activated BM-DCs of the indicated genotype. Where indicated, rIL-15 was added at 100 ng/ml. Controls: DC + NK; DC + LPS; NK + LPS; DC; NK; in (A), ± rIL-15.

(C) Immunocytofluorescence of WT and mutant NK cells treated or not with 200 U/ml rIFN-β for 3 hr. Where indicated, BFA was added. IL-15 (green) was detected with an indirect antibody staining, whereas the membrane (red) was counterstained with cholera toxin B.

(D) IFN-γ production by WT or IL-15-deficient NK cells cocultured with LPS-activated WT or IL-15-deficient BM-DCs. Controls: DC + NK; DC + LPS; NK + LPS; DC; NK.

(E) IFN-γ production by WT NK cells stimulated with rIL-2 (1 ng/ml), rIL-18 (120 pg/ml), and rIFN-β (200 U/ml) in the presence of the indicated neutralizing antibodies (or isotype control).

(A, D, and E) n ≥ 3. Statistical significance was determined with ANOVA followed by Dunnett's multiple comparison test. (B) n ≥ 3. Statistical significance was determined with a two-tailed t test. (C) n = 2. Error bars depict SEM. **, $p < 0.01$; ****, $p < 0.0001$; ns, not significant.

See also Figures S5 and S6.

IFN-γ release was severely impaired (Figure 6D). This was not due to intrinsic unresponsiveness of *Il15ra*$^{-/-}$ NK cells, because a type I IFN-independent stimulation with a well-described combination of activating cytokines produced during viral infections, IL-12 and IL-18 (Andrews et al., 2003; Fehniger et al., 1999; Robinson et al., 1997), resulted in an adequate release of IFN-γ by *Il15ra*$^{-/-}$ NK cells, far larger than when cells received a type I IFN-dependent stimulation (Figure 6D). This is in agreement with published reports of functional integrity of *Il15*$^{-/-}$ and *Il15ra*$^{-/-}$ NK cells (Sun et al., 2009). Consistent with the defective response of *Il15ra*$^{-/-}$ NK cells to IL-2, IL-18, and IFN-β, clear activation impairment was also observed in DC-NK cell cocultures when both cell types were IL-15Rα-deficient (Figure 6E).

The observation that IL-15 could be self-produced by NK cells opened the question whether NK cell-derived IL-15 could only be effective autocrinally, via IL-15Rα-mediated *cis* presentation, or also paracrinally, through *trans* presentation between different NK cells. To investigate this question, WT and *Il15ra*$^{-/-}$ NK cells were cocultured at different ratios and activated with IL-2, IL-18, and IFN-β. We reasoned that IL-15Rα-deficient NK cells would produce IFN-γ only if IL-15 could be *trans*-presented by juxtaposed IL-15Rα-competent NK cells. Moreover, the efficiency of *trans* presentation (but not *cis* presentation) would be mitigated at low densities of IL-15Rα-sufficient cells. We found that, independently of the ratio between WT and *Il15ra*$^{-/-}$ NK cells, only WT NK cells could produce IFN-γ (Figure 6F), indicating that NK cell-derived IL-15 can only operate in an autocrine way via *cis* presentation.

NK Cell Cytotoxic Activity Requires IFN-β and IL-15 but Not IL-2 or IL-18

In addition to IFN-γ production, NK cells are endowed with another major function, namely cytotoxic activity against

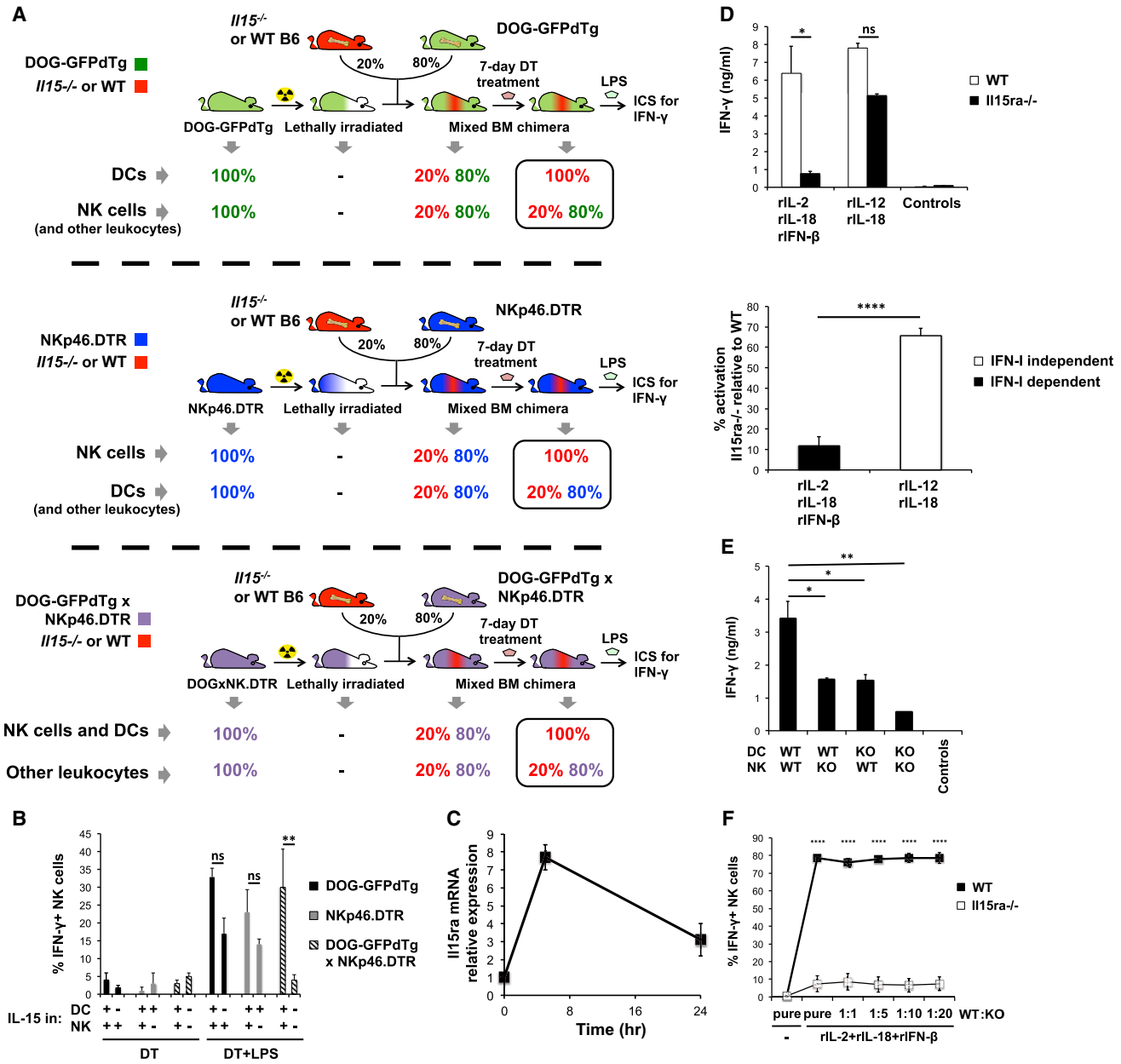

Figure 6. IL-15 Triggered by IFN-β Can Be Presented by IL-15Rα in *cis* by NK Cells and in *trans* by DCs and Is Required for NK Cell Activation In Vivo

(A) Experimental design for (B). Chimeric mice were generated and treated with DT as shown to obtain the following phenotypes: IL-15-deficient DCs and IL-15-sufficient (GFP$^+$) NK cells using DOG-GFPdTg recipients (top panel); IL-15-sufficient DCs and IL-15-deficient (GFP$^-$) NK cells using NKp46.DTR recipients (middle panel); IL-15-deficient DCs and IL-15-deficient (GFP$^-$) NK cells using DOG-GFPdTg × NKp46.DTR recipients (bottom panel). All recipient mice were reconstituted with BM cells for 80% from donors of the same genotype as the recipient and for 20% from *Il15*$^{-/-}$ (or WT as a control) animals. Chimeric mice were treated with DT for 1 week to deplete IL-15-sufficient DCs, NK cells, or both, allowing IL-15-deficient progenitors to proliferate and replenish their niches. One day after the last DT injection, mice were challenged with LPS, and the percentage of activated (IFN-γ$^+$) NK cells was determined at 5 hr.

(B) Results of the experiment depicted in (A).

(C) *Il15ra* mRNA expression in sorted WT NK cells (CD49b$^+$CD3$^-$) at the indicated time points upon rIFN-β exposure (200 U/ml). Data are expressed as relative expression over the basal level measured in unstimulated cells at the same time point.

(D) Sorted NK cells (CD49b$^+$CD3$^-$) of the indicated genotype were activated with either rIL-2 (1 ng/ml) + rIL-18 (120 pg/ml) + rIFN-β (200 U/ml) or rIL-12 (100 pg/ml) + rIL-18 (120 pg/ml). After 18 hr, IFN-γ was measured in cell-free supernatants. Absolute values (top) or relative ratios (*Il15ra*$^{-/-}$ over WT, bottom) are shown. Removing any recombinant cytokine from either cocktail results in an ineffective stimulation (controls).

(E) BM-DCs and NK cells from WT or *Il15ra*$^{-/-}$ (KO) mice were cocultured in the presence of LPS for 18 hr before measuring IFN-γ. Controls: DC + NK; DC + LPS; NK + LPS; DC; NK.

(legend continued on next page)

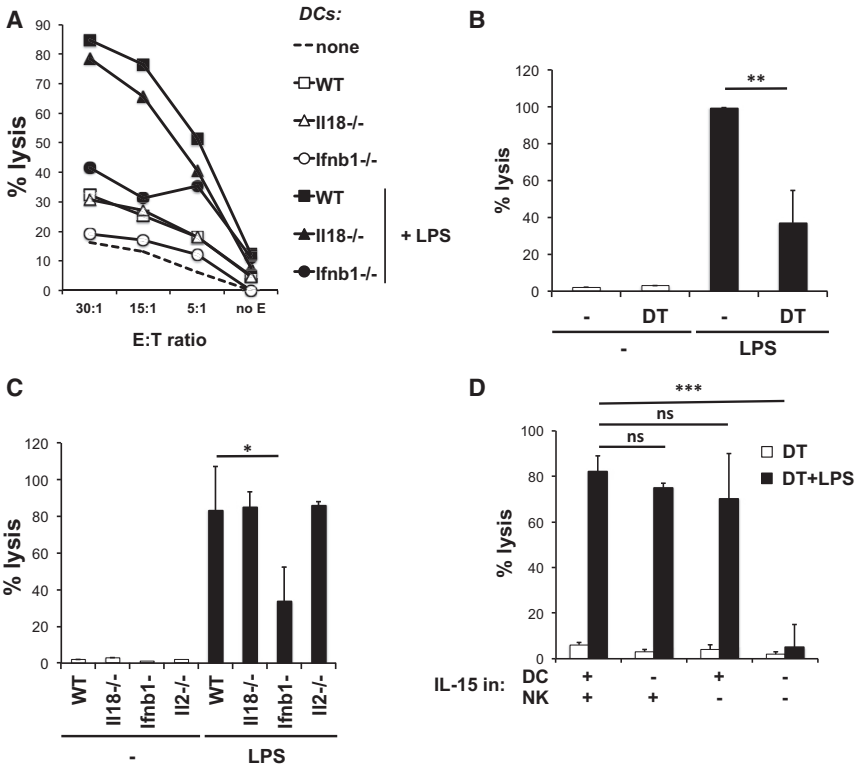

Figure 7. NK Cell Cytotoxic Activity Requires IFN-β and *cis*- or *trans*-Presented IL-15

(A) BM-DCs of the indicated genotype, either untreated or stimulated by LPS, were cocultured with graded numbers of WT NK cells for 6 hr. Afterward, YAC-1 target cells were added for 3 additional hr to measure NK cell-mediated lysis. One representative experiment out of six is shown.

(B–D) MHC class I-sufficient and deficient splenocytes labeled with different concentrations of CFSE were injected i.v. at 1:1 ratio together with LPS to activate NK cells. One day later, spleen cells were analyzed by flow cytometry and the ratio between sensitive (MHC-I-deficient) and resistant (MHC-I-sufficient) target cells was determined. (B) In vivo cytotoxic activity of NK cells in CD11c.DOG mice treated or not with DT to deplete DCs. (C) In vivo cytotoxic activity of NK cells in the indicated knockout mice. (D) In vivo cytotoxic activity of NK cells in mixed BM chimera mice (strategy illustrated in Figure 6A) bearing *Il15*$^{-/-}$ DCs and *Il15*$^{+/+}$ NK cells, *Il15*$^{+/+}$ DCs and *Il15*$^{-/-}$ NK cells, or *Il15*$^{-/-}$ DCs and *Il15*$^{-/-}$ NK cells.

(B–D) n = 6 mice per group from three independent experiments. Statistical significance was determined with a one-tailed t test (B) or with ANOVA followed by one-tail Dunnett's multiple comparison test (C and D). Error bars depict SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

missing- or altered-self targets (Lanier, 2005). Therefore, we asked whether IL-2, IL-18, and IFN-β had a role in promoting NK cell cytotoxicity as well. The NK cell lytic capacity was first investigated in vitro. As shown in Figure 7A, NK cell cytotoxicity was poor in the absence of DCs. The addition of DCs greatly boosted NK cell-mediated killing. IL-2 and IL-18 displayed no role in the DC-mediated enhancement of NK cell cytotoxic activity. By contrast, IFN-β was essential.

We then analyzed the requirements to activate the NK cell cytotoxic activity in vivo by measuring the selective killing of β-2 microglobulin-deficient target cells. First, we aimed at confirming the finding that DCs were required to induce the NK cell cytotoxic function upon LPS administration (as observed for IFN-γ production). As shown in Figure 7B, the capacity of NK cells to efficiently kill target cells was in fact dependent on the presence of DCs. Second, we analyzed the cytotoxic activity of NK cells in IL-2-, IL-18-, or IFN-β-deficient mice. We observed that the cytotoxic activity of NK cells was completely preserved following LPS injection in IL-18-deficient and IL-2-deficient mice but not in IFN-β-deficient mice (Figure 7C). Because the role of IFN-β was confirmed both in vitro and in vivo, we asked whether that was mediated by IL-15. To this end, we performed a cytotoxicity experiment in mixed BM chimeric mice, in which DCs, NK cells, or both DCs and NK cells had been deprived of the capacity to produce IL-15 (similar to the experimental setting depicted in Figure 6A). Interestingly, the lytic activity of NK cells was totally inhibited only when both DCs and NK cells were unable to produce IL-15 (Figure 7D). This is in line with our findings on the role of IL-15 for NK cell-derived IFN-γ release (Figure 6B), once again indicating that IL-15 was required to activate NK cell cytotoxic functions, regardless of the source (DCs or NK cells).

In conclusion, the cytotoxic activity of NK cells in LPS-induced inflammatory conditions depends on conventional DCs, IFN-β and IL-15. IL-15 can be either *trans*-presented by DCs or *cis*-presented by NK cells.

DISCUSSION

NK cell activation had long been regarded as a cell autonomous event. The balance of signals arising from activating and inhibitory receptors, engaged by transformed or infected cells, would ultimately determine the magnitude of NK cell responses (Horowitz et al., 2012). This oversimplified view was first called into question by the finding that DCs can potently stimulate the

(F) NK cells sorted (CD49b^{+} CD3^{-}) from IL-15-sufficient Ubiquitin. GFP (WT) or *Il15ra*$^{-/-}$ mice were mixed at different ratios and stimulated with rIL-2 (1 ng/ml) + rIL-18 (120 pg/ml) + rIFN-β (200 U/ml). After 5 hr (2 hr with cytokines and 3 hr with brefeldin A), the fraction of IFN-γ-secreting cells was determined by intracellular staining.

(B) n = 4–6 mice per group from three independent experiments. (C and D) n ≥ 3. (E and F) n = 4. Statistical significance was determined with ANOVA followed by Sidak's ([B], [D] top, [F]) or Dunnett's (E) multiple comparison tests. A two-tail t test was used to analyze (D) bottom. Error bars depict SEM. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$; ns, not significant.

See also Figures S5, S6, and S7.

antitumor functions of NK cells (Fernandez et al., 1999). Details of such interaction began to emerge, and the figure being revealed dramatically altered our understanding of NK cell biology (Newman and Riley, 2007). Now, parallels between NK cells and T cells are being drawn, as illustrated by the proposed concept of DC-mediated NK cell priming (Chaix et al., 2008; Koka et al., 2004; Lucas et al., 2007), echoing naive T-cell priming. Essentially, a shift of focus from the innate features to the lymphocytic nature of NK cells has occurred. However, differently from T cells, details of DC-NK cell interactions are scattered and hard to reconcile (Newman and Riley, 2007). Several molecular signals have been implicated in priming and/or activation, two nonoverlapping events, yet often blearily distinguished in the literature. Nonetheless, to date, a set of signals not only necessary but also sufficient for NK cell activation has not been identified, especially in response to Gram-negative bacteria.

We proved that DCs are the accessory cells required not simply for NK cell priming, as previously proposed (Lucas et al., 2007), but in fact for direct NK cell activation. Indeed, IFN-γ was immediately released, even in the absence of restimulation (Figure 1A), and that was dependent on the presence of DCs (Figure 1B). We ruled out the possibility that NK cells are able to sense LPS directly (Figure 2B). Indeed, several studies reported a role for autonomous recognition of TLR agonists by NK cells (Becker et al., 2003; Chalifour et al., 2004; Hart et al., 2005; Lauzon et al., 2006; Sivori et al., 2004, 2006, 2007; Tsujimoto et al., 2005). This is not the case for TLR4, as TLR4-deficient NK cells displayed no defect in IFN-γ release (Figure 2B). By contrast, TLR4 was required on DCs in vitro and in vivo, highlighting the accessory cell-dependent nature of NK cell activation in this context (Figures 2A and 4B).

Then, we showed that full IFN-γ release by NK cells is elicited by three DC-derived cytokines secreted in response to LPS: IL-2, IL-18, and IFN-β (Figures 2 and 4). A crucial role of IFN-β is to trigger IL-15 release by both DCs and NK cells. In turn, IL-15 can act in a dual fashion: paracrinally (*trans*-presented by DCs to NK cells), as previously shown (Lucas et al., 2007), but strikingly also in an autocrine way, via *cis* presentation by an NK cell to itself (Figures 5, 6, and 7D). IL-15 is preassembled in complex with IL-15Rα in the endoplasmic reticulum/Golgi compartment (Mortier et al., 2008). Then, IL-15Rα "presents" IL-15 to IL-2/15Rβ/γ, initiating intracellular signaling (Koka et al., 2004). Such a unique mechanism can operate both in *trans* (IL-15Rα and IL-2/15Rβ/γ are expressed on two different cells, usually of different type; Brilot et al., 2007; Koka et al., 2004) and in *cis* (IL-15Rα and IL-2/15Rβ/γ are expressed on the same cell; Olsen et al., 2007; Rowley et al., 2009). The role of IL-15 *trans* presentation from DCs to NK cells is seen as largely prevalent during NK cell activation (Koka et al., 2004; Lucas et al., 2007; Mortier et al., 2008). However, in line with our observation that IFN-β-stimulated NK cells express both IL-15 and IL-15Rα (Figures 5C, 6C, S5, and S6), we found that *cis* and *trans* presentation are codominant mechanisms in NK cell activation. Differently from the prevalent view, we show that, in the absence of IL-15 *trans* presentation, NK cells can still undergo activation, thanks to their IL-15 *cis*-presenting activity. Indeed, only when both DCs and NK cells were IL-15 (or IL-15Rα) deficient was NK cell activation severely impaired (Figures 5D, 6B, and 6E). Conversely, when IL-2, IL-18, and IFN-β were used to stimulate NK cells (Figures 5E, 6D, and 6F), IL-15 presentation could solely take place in *cis*. It is worth noting that there have been other reports of IL-15Rα upregulation in NK cells, such as in response to MCMV (French et al., 2006). However, because IL-15 neither is secreted by DCs as free protein nor dissociates from IL-15-IL-15Rα complexes (Mortier et al., 2008), there is no free soluble IL-15 available to bind to unloaded IL-15Rα under physiological conditions. Consequently, the functional significance of IL-15Rα expression by NK cells remained elusive. We found that IL-15 can be expressed by NK cells in an IL-15Rα-dependent fashion (Figures 5, 6, and S6), thereby providing a rationale for the observed IL-15Rα expression in NK cells. Altogether, our findings call for a revaluation of the contribution of IL-15 *cis* presentation in DC-mediated NK cell activation and possibly in other biological processes relying on IL-15. Whereas our discovery of physiological IL-15 *cis* presentation focused on NK cell biology, it may likely impact on several other IL-15-dependent processes as long as type I IFNs are involved.

The short-tailed IL-2Rα and IL-15Rα are not thought to be endowed with any signaling properties, although this notion has been questioned, at least for IL-15Rα (Pohl et al., 2011; Castro et al., 2011). However, the simultaneous requirement for IL-2 and IL-15, both engaging the IL-2/15Rβ/γ-STAT5 axis, to achieve an efficient DC-mediated NK cell activation (Figures 2H, 4B, 5, 6, and S2) points to nonredundant signaling properties of the two cytokines. This is also supported by the finding that IL-15 but not IL-2 contributes to NK cell cytotoxicity in response to LPS (Figure 7). The conundrum is even more intriguing in view of a recent study that could not detect any substantial signaling divergence between IL-2 and IL-15 (Ring et al., 2012), leaving observers to speculate that only receptor occupancy and/or signaling context may solve the paradox (Ikemizu et al., 2012). However, the case of DC-mediated NK cell activation, where the signaling contexts of IL-2 and IL-15 are similar, increases the challenge and provides fertile ground for future investigation.

Why IL-15 is controlled downstream of type I IFNs (Zhang et al., 1998) rather than being directly elicited by PAMP recognition as most myeloid cell-derived cytokines is an intriguing question. By uncovering that lymphoid cells, such as NK cells, can also produce IL-15, this missing direct link between pattern recognition and IL-15 release appears more reasonable. Another point is that IL-15 contributes not only to activation but also to survival of NK cells and memory $CD8^+$ T cells (Berard et al., 2003; Brilot et al., 2007; Burkett et al., 2004; Huntington et al., 2009; Ranson et al., 2003), that is, cytotoxic lymphocytes involved in immediate first-line responses. Large amounts of type I IFNs are produced during infections by both immune and nonimmune cells (Sen, 2001). It is tempting to link these two observations by speculating that, during infections, any type I IFN-secreting cell (not only DCs) might promote NK cell survival by eliciting IL-15, with the final outcome of strengthening the immune response. Notably, this would be different from steady-state homeostasis of NK cells, which is a noncell autonomous event (in that IL-15-competent NK cells die in an IL-15-deficient recipient) and does not depend on the presence of type I IFNs (Huntington et al., 2009; Ma et al., 2006). The mechanism of IL-15-dependent survival would also be different from

IL-15-dependent activation, as we showed how the latter is more strictly dependent on DCs (Figures 5, 6, and 7). Nevertheless, IL-15-dependent survival and activation would conceivably share portions of their underlying molecular mechanisms. In this light, the dependence of IL-15 production from type I IFNs, which would be reasonable for the dynamic of NK cell survival during infections, might be conserved during the process of NK cell activation, where direct IL-15 production by DCs in response to PAMPs would otherwise be expected, as it occurs for most cytokines. We speculate that another reason for the type I IFN-dependence of IL-15 might be its previously unappreciated *cis* presentation, which we uncovered here (Figures 5, 6, and 7). Indeed, because NK cells cannot sense LPS directly (Figure 2B), they would not be able to produce IL-15, was it placed directly downstream of PRRs. Conversely, the regulation through a soluble mediator (such as IFN-β) allows even cells not equipped to directly recognize PAMPs (such as NK cells) to produce cytokines (such as IL-15) essential for immune responses.

IL-1β and IL-18 are usually released in a two-step process—priming and inflammasome activation (Schroder and Tschopp, 2010)—and LPS is thought to provide only priming. However, there are instances when LPS alone is able to trigger the secretion of biologically active IL-1 and IL-18. This is the case, for instance, of human monocytes (Netea et al., 2009). We have also shown that ultrapure rough LPS (the type used in this study) alone is able to trigger ASC-dependent inflammasome activation and subsequent IL-1β and IL-18 production in DCs (Zanoni et al., 2012).

Finally, we also investigated the molecular requirements of productive DC-NK cell interactions in terms of enhancement of cytotoxic responses. DC-derived IL-2 at physiological concentrations was previously shown to be dispensable for eliciting cytotoxic responses in vitro (Granucci et al., 2004). In the present study, we confirmed this observation in vivo, and we also report that IL-18 is similarly dispensable (Figure 7). Conversely, in agreement with the literature (Nguyen et al., 2002), we found that DC-derived IFN-β strongly boosts the cytotoxic responses of NK cells. The effect of IFN-β may be partially direct, but surely, its indirect effect through the induction of IL-15 is critical (Figure 7D). Again, as already seen for IFN-γ secretion (Figures 5D, 6B, and 6E), the lack of IL-15 on DCs can be functionally complemented by IL-15 *cis* presented by NK cells (Figure 7D).

Our results shed light on the mechanisms underlying NK cell activation in vivo in response to *E. coli* and its major bacterial PAMP, LPS. A precise understanding of the biological program governing NK cell responses might provide a framework for next-generation immunotherapeutic strategies based on the manipulation of NK cell functions.

EXPERIMENTAL PROCEDURES

Mice

All mice, housed under specific pathogen-free conditions, had been on a B6 background for at least 12 generations and were used at 7–12 weeks of age. *Il12a*$^{-/-}$ mice were B6 albino. The genetic background of B6 albino mice is identical to that of B6 mice, as confirmed by The Jackson Laboratory. Experiments were performed using protocols approved by the Institutional Animal Care and Use Committee of the University of Milano-Bicocca.

WT animals were supplied by Harlan Italy. *Il1r1*^{tm1Inx} (*Il1r1*$^{-/-}$), *Il2*^{tm1Hor} (*Il2*$^{-/-}$), *Il18r1*^{tm1Aki} (*Il18r1*$^{-/-}$), *Il18*^{tm1Aki} (*Il18*$^{-/-}$), and *Ticam1*^{Lps2} (*Ticam1*$^{-/-}$) mice were purchased from The Jackson Laboratory. *Il12a*^{tm1Jm} (*Il12a*$^{-/-}$) and *Il15ra*^{tm1Ama} (*Il15ra*$^{-/-}$) mice were kindly provided by L. Romani (University of Perugia, Italy) and S. Bulfone-Paus (University of Manchester, UK). *Il15*^{tm1Imx} (*Il15*$^{-/-}$) animals were from Taconic. *Myd88*^{tm1Aki} (*Myd88*$^{-/-}$) and *Tlr4*^{tm1Aki} (*Tlr4*$^{-/-}$) mice were provided by S. Akira (IFReC, Japan). *Ifnb1*^{tm1Tl} (*Ifnb1*$^{-/-}$) mice were supplied by S. Weiss (Helmholtz Centre for Infection, Germany). Ubiquitin.GFP (Ikawa et al., 1998) and *B2m*^{tm1Unc} (*B2m*$^{-/-}$) mice were obtained from M. Battaglia (San Raffaele Telethon Institute for Gene Therapy, Italy); NKp46.DTR (Walzer et al., 2007) mice, in which NK cells are labeled with GFP and express the DTR for conditional DT-mediated depletion, were a kind gift from E. Vivier (Centre d'Immunologie de Marseille-Luminy, France). CD11c.DOG mice have already been described (Hochweller et al., 2008). Briefly, this transgenic model expresses the DTR under the control of a cloned CD11c promoter, allowing selective depletion of DCs (CD11c^{high}) upon DT treatment. The CD11c promoter region cloned in CD11c.DOG mice is longer than in former CD11c.DTR/GFP mice (Jung et al., 2002), resulting in a highly faithful DTR expression, which in turn allows DT-mediated ablation of DCs for an extended time without toxicity.

To deplete DCs in experiments where LPS was delivered subcutaneously (s.c.) (Figure 1B), DT was injected s.c. 4 hr before LPS challenge, as DC depletion was already highly effective and specific at this time point (Figure S1). Conversely, when LPS was delivered i.v., CD11c.DOG mice received a single intraperitoneal (i.p.) injection of 16 ng DT (Sigma-Aldrich) per gram of body weight (gbw) at day −1.

In order to largely restrict a cytokine deficiency to DCs, mixed BM chimeras were generated (Figure 4A) as follows. CD11c.DOG/ubiquitin.GFP double-transgenic (DOG-GFPdTg) mice were exposed to lethal (950 rad) whole-body irradiation and reconstituted with 5 × 10^6 BM cells and then allowed to recover for at least 2 months with antibiotics before being used for experiments. Control nonreconstituted mice did not survive, confirming the lethality of irradiation. Twenty percent of donor BM cells came from mice deficient for the cytokine under examination, whereas the remaining 80% came from DOG-GFPdTg mice. A similar strategy was used to generate mixed BM chimeras based on NKp46.DTR donor cells, as described in the main text and depicted in Figure 6A. Mixed BM chimeras received a daily i.p. injection of 16 ng DT/gbw for 7 days. This allowed enough time for niche replenishment by differentiating precursors, restricting the repopulation potential to the DT-insensitive (and deficient for a selected cytokine) fraction only.

Cells

All cells were cultured in Iscove's modified Dulbecco's medium (IMDM)-10 complete medium: IMDM, 10% heat-inactivated fetal bovine serum (FBS) (EuroClone), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol (Sigma-Aldrich).

BM-DCs were generated by culturing BM precursors, flushed from femurs, in granulocyte-macrophage colony-stimulating factor-supplemented medium for 8–10 days, as described (Granucci et al., 2001; Inaba et al., 1992).

NK cells were purified from red blood cell (RBC)-lysed splenocytes by magnetic-activated cell sorting (MACS) positive selection using CD49b (DX5) microbeads (Miltenyi Biotec). Purity was assessed by fluorescence-activated cell sorting (FACS) and was routinely between 93% and 96%. Alternatively, NK cells were first enriched by MACS negative selection for Ly-6G (1A8), CD19 (6D5), and CD3ε (145-2C11) using biotinylated antibodies (Abs) and streptavidin microbeads and then stained with anti-CD49b and sorted with a FACSAria II (BD Biosciences). Purity was consistently greater than 99.5%. MACS-selected and FACS-sorted NK cells produced similar results in our experiments. Where indicated, NK cells were identified and sorted as CD49b$^+$CD3$^-$CD122$^+$ cells.

YAC-1 cells were purchased from the American Type Culture Collection. Human peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-anticoagulated blood using standard Ficoll density gradient centrifugation. PBMCs were cryopreserved in freezing medium (10% DMSO in FBS). Upon thawing, cells were allowed to rest for 6 hr in complete medium before staining, sorting, and stimulation. Human studies were approved by the ethical committee of the University of Milano-Bicocca.

DC-NK Cell Cocultures

BM-DCs (8×10^4/well) and NK cells (8×10^4/well for IFN-γ release assays; scaling numbers for cytotoxicity assays according to the effector:target ratio) were cocultured in flat-bottom 96 well plates in the presence or absence of the following reagents: ultrapure TLR-grade Re-form LPS from *E. coli* serotype R515 (1 μg/ml)—shown to be free of any contaminating lipoproteins or peptidoglycan, neutralizing anti-IL-1β (B122), anti-IL-1R1 (35F5), anti-IL-18Rβ (TC30-28E3), anti-IL-12(p40/p70) (C17.8), and proper isotype controls from BD Biosciences; neutralizing anti-IL-18 (D048-3) from MBL International; neutralizing anti-CD70 (FR70) from eBioscience; neutralizing anti-IL-15 (AF447) and anti-IL-15Rα (AF551) from R&D Systems; recombinant (r) IL-1RA (1 μg/ml), rIL-18BPd/Fc chimera (1 μg/ml), rhIgG1 Fc (1 μg/ml), rIL-12, rIL-15, and rIL-18 from R&D Systems; rIFN-β from PBL InterferonSource; and rIL-2 from Peprotech. All neutralizing Abs and relevant isotype controls were used at 10 μg/ml and were purchased as no azide/low endotoxin format. Refer to figure legends for concentrations of recombinant cytokines.

Abs and recombinant proteins aimed at neutralizing cytokines (IL-1RA and IL-18BPd/Fc chimera) were added 30 min prior to stimulation with LPS. Activating recombinant cytokines (rIL-2, rIL-18, rIFN-β, and rIL-15) were added at the time of LPS stimulation.

ELISA

Cell-free supernatants were collected from DC-NK cell cocultures after 18 hr and were analyzed using an IFN-γ DuoSet ELISA Kit (R&D Systems). We have previously demonstrated that IFN-γ is exclusively produced by NK cells in DC-NK cell cocultures (Granucci et al., 2004).

Immunocytofluorescence

NK cells were cultured in the presence of IFN-β (200 U/ml) for 3 hr before being cytospun onto glass slides. After fixation with 4% paraformaldehyde and permeabilization with perm/blocking buffer (0.1% Triton X-100/0.2% BSA in PBS), cells were stained with a goat anti-mouse IL-15 (10 μg/ml, L-20, Santa Cruz) followed by a secondary incubation with Alexa Fluor 488-conjugated chicken anti-goat immunoglobulin G (2 μg/ml, Life Technologies), diluted in blocking buffer. Cell membranes were counterstained with Alexa Fluor 555-conjugated Cholera Toxin Subunit B (CTB, Life Technologies). A TCS SP2 confocal microscope (Leica) was used to acquire images. At least five fields were acquired for each slide.

Quantitative Real-Time PCR

FACS-sorted CD11c$^+$ BM-DCs (from day 10 cultures), ex vivo CD49b^{high}CD3$^-$ murine NK cells (from spleens), or NKp46$^+$CD56$^+$CD3$^-$ human NK cells (from PBMCs) were cultured in the presence of species-matched rIFN-β (200 U/ml) for the time shown in figures. Cells were lysed with TRIzol (Life Technologies), and total RNA was extracted with the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. A NanoDrop spectrophotometer (Thermo Scientific) was used to quantify RNA and to assess its purity. RNA was retrotranscribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Then, 10 ng cDNA was amplified using the Power SYBR Green PCR Master Mix (Applied Biosystems) in a 7500 Fast Real-Time PCR System (Applied Biosystems), and data were analyzed using the built-in SDS software.

The sequences of mouse primer pairs were: *Il15*, 5′-CATTTTGGGCTGTGTCAGTGT-3′ and 5′-ACTGGGATGAAAGTCACTGTCAGTG-3′; *Il15ra*, 5′-GCCTCAAGTGCATCAGAGACC-3′ and 5′-ACCTTTGGTGTCACTACTGTTGGC-3′; and *18S*, 5′-CGAAAGCATTTGCCAAGAAT-3′ and 5′-AGTCGGCATCGTTTATGGTC-3′. The sequences of human primer pairs were: *IL15*, 5′-TGTTCCATCATGTTCCATGC-3′ and 5′-TCCACGATGCCTCCTACAA-3′; *IL15RA*, 5′-CAAGCTGTAGCTCTTGACCCA-3′ and 5′-GCTACTGCTGCTGCTGCTC-3′; and *ACTB*, 5′-GTTGTCGACGACGAGCG-3′ and 5′-GCACAGAGCCTCGCCTT-3′. 18S and β-actin were used as endogenous references for relative quantification with the ΔΔCt method.

In Vitro Cytotoxicity Assay

NK cell cytotoxicity was quantitated by a dye-release assay coupled to time-resolved fluorescence using the DELFIA EuTDA Cytotoxicity Reagents (PerkinElmer; Blomberg et al., 1996). Briefly, 5×10^6 YAC-1 target cells resuspended in 1 ml IMDM-10 were labeled with 5 μl BATDA reagent (the acetoxymethyl ester of the fluorescence-enhancing organic molecule TDA) at 37°C for 30 min. Within cells, the ester bonds are hydrolyzed to form the hydrophilic TDA, which no longer passes the membrane. Cells were then washed four times with PBS and 2×10^4 targets per well were added to a 6-hr-long DC-NK cell coculture for an additional 3 hr. A control of TDA maximal release was obtained by incubating target cells with DELFIA lysis solution, whereas spontaneous release was measured on target cells alone. Fifty microliter cell-free supernatants, containing the TDA released by killed cells, were mixed to 150 μl DELFIA europium solution and incubated for 10 min with shaking. Time-resolved fluorescence of the Eu:TDA chelate, correlating with the number of lysed cells, was measured using a Victor3 plate reader (PerkinElmer). Medium background was subtracted from all conditions. Results are shown as the percentage of target cell lysis relative to the maximum and spontaneous release controls.

In Vivo NK Cell Activation and IFN-γ Secretion

To activate NK cells, age-matched mice were injected i.v. with 2 μg LPS/gbw, except for the experiments depicted in Figure 1, where mice were injected s.c. in the anterior footpad with 1 μg LPS/gbw. Alternatively, in Figures 3A–3C, mice were challenged with 3×10^7 *E. coli* colony-forming units (CFU). Mice were euthanized 2.5 hr and 4 hr before the 5 hr and 18 hr time points, respectively, to culture RBC-lysed splenocytes in the presence of brefeldin A (BFA; 10 μg/ml; Sigma-Aldrich) for the remaining time. This allowed measuring direct IFN-γ release by NK cells in the absence of artificial restimulation. Intracellular staining was performed using Cytofix/Cytoperm reagents (BD Biosciences) according to the manufacturer's instructions, with the following Abs: anti-CD49b (DX5 or HMα2); anti-CD3ε (145-2C11); anti-CD11c (HL3); and anti-IFN-γ (XMG1.2) (or its isotype control).

Bacterial Challenge

Amp^r-E. coli (strain DH5α) was grown to midlogarithmic phase in Luria broth (LB) medium and frozen at −80°C in glycerol stocks. Before infection, *E. coli* was regrown in LB/ampicillin medium until optical density 600 = 0.6. Mice were challenged with 3×10^7 bacteria, as calculated from the linear relationship with turbidity and checked by the number of CFU grown after 24–36 hr at 37°C. Spleens were collected and homogenized in 0.2% Triton X-100 18 hr later. Bacterial titers were determined by serially diluting homogenates on LB agar plates and counting CFU after 24–36 hr at 37°C.

In Vivo Cytotoxicity Assays

Splenocytes isolated from *B2 m$^{-/-}$* mice were used as NK cell targets in vivo, whereas the WT counterparts served as a control (Regner et al., 2011). The two populations were tracked and distinguished upon loading with two different concentrations (0.75 μM or 0.075 μM) of carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies). Labeled cells were adoptively transferred at a 1:1 ratio (checked by flow cytometry before injection) along with 2 μg LPS/gbw to activate NK cells. Target and control cells were recovered 24 hr later from the spleen, and the ratio between the two populations was assessed by flow cytometry.

Statistical Analysis

Results are expressed as mean ± SEM from multiple independent experiments. In in vitro studies, n refers to the number of independent experiments. In in vivo studies, n refers to the number of animals per condition.

Hypotheses were tested with two-tail t tests in single pairwise comparisons or multiple orthogonal comparisons. ANOVA posthoc tests were used for correction of multiple nonorthogonal comparisons: Dunnett's when multiple conditions were tested against a single control condition; Sidak's when a subset of conditions was selected for pairwise comparisons; and Tukey's when all possible combinations were tested. One-sample two-tail t tests were used for comparisons against a fixed value (100%). Dependent samples were analyzed with paired t tests or repeated-measures ANOVA. p values, calculated with Excel (Microsoft) or Prism (Graphpad), are coded by asterisks: <0.05 (*), <0.01 (**), <0.001 (***), <0.0001 (****); ns, not significant.

Figure legends report the type of error bars, n, and the hypothesis test used to compare differences.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.08.021.

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