A Contribution of Mouse Dendritic Cell–Derived IL-2 for NK Cell Activation

Francesca Granucci,[1] Ivan Zanoni,[1] Norman Pavelka,[1]
Serani L.H. van Dommelen,[2] Christopher E. Andoniou,[2]
Filippo Belardelli,[3] Mariapia A. Degli Esposti,[2] and Paola Ricciardi-Castagnoli[1]

[1]*Department of Biotechnology and Bioscience, University of Milano-Bicocca, 20126 Milan, Italy*
[2]*Immunology and Virology Program, Centre for Ophthalmology and Visual Sciences, The University of Western Australia and Centre for Experimental Immunology, Lions Eye Institute, Perth 6009, Western Australia, Australia*
[3]*Laboratory of Virology, Istituto Superiore di Sanità, 00100 Rome, Italy*

Abstract

Dendritic cells (DCs) play a predominant role in activation of natural killer (NK) cells that exert their functions against pathogen-infected and tumor cells. Here, we used a murine model to investigate the molecular mechanisms responsible for this process. Two soluble molecules produced by bacterially activated myeloid DCs are required for optimal priming of NK cells. Type I interferons (IFNs) promote the cytotoxic functions of NK cells. IL-2 is necessary both in vitro and in vivo for the efficient production of IFNγ, which has an important antimetastatic and antibacterial function. These findings provide new information about the mechanisms that mediate DC–NK cell interactions and define a novel and fundamental role for IL-2 in innate immunity.

Key words: NK cells • dendritic cells • innate immune response • interleukin 2 • interferon γ

Introduction

After exposure to microbial stimuli, DCs undergo a process of gene transcription reprogramming that controls an ordered sequence of maturation events (1–3) with the sequential acquisition of specific immune-regulatory activities (4, 5). These events include an initial profound cytoskeleton rearrangement correlated with the loss of antigen internalization (6), the subsequent enhancement of antigen processing efficiency (7), the loss of T cell tolerization function (8), and the acquisition of the ability to prime antigen-specific $CD4^+$ and $CD8^+$ T cell responses (9). During the maturation process, DCs produce cytokines, chemokines, and cell surface molecules, such as costimulatory molecules, with strictly defined kinetics (10). Recent studies have focused on the function of DCs during the early phases of the immune response, and a predominant role for DCs in activation of NK cells has been described (11–14).

NK cells are specialized lymphocytes of the innate immune system capable of eliciting responses against pathogen-infected and tumor cells. They are activated during the early phases of an immune response, a few hours after infection. The functions of NK cells are regulated by a balance of activating and inhibiting signals. These signals are transmitted by inhibitory receptors, which bind class I major histocompatibility complex (MHC) molecules, and activating receptors, which bind ligands on tumors and pathogen-infected cells. Other than surface receptors, cytokines, such as IL-2, IL-12, IL-18, and type I IFNs, have been shown to promote NK cell priming (15).

The biological relevance of NK cell activation mediated by DCs during bacterial infections resides mainly in the secretion of IFNγ (16), which represents the principal phagocyte-activating factor (16, 17). With regards to antitumor activities, the interaction between activated DCs and NK cells has been described to increase the efficiency of NK cell antitumor effector functions both in vitro and in vivo in two independent experimental models (14, 18). Despite the confirmed ability of DCs to prime NK cell bactericidal and antitumor responses, the molecular mechanisms responsible for DC-mediated NK cell activation remain to be elucidated.

A classical method to activate NK cells in vitro and to increase their antitumor effectiveness involves culturing NK cells in the presence of IL-2. However, this cytokine has never been considered important in vivo for NK cell–mediated antitumor or antimicrobial responses as it was believed that IL-2 was exclusively produced by T cells during the late, anti-

F. Granucci and I. Zanoni contributed equally to this work.

Address correspondence to Paola Ricciardi-Castagnoli, Dept. of Biotechnology and Bioscience, University of Milano–Bicocca Piazza della Scienza, 2, 20126 Milano, Italy. Phone: 39-02-64483559; Fax: 39-02-64483552; email: paola.castagnoli@unimib.it

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gen-specific phase of the immune response, when the peak of NK cell activation was already exhausted (19). On the other hand, we have observed recently that in the mouse not only T cells but also DCs are able to produce IL-2 (1). DCs acquire this capacity 1 h after stimulation with bacteria (1) or bacterial cell products (20), a timing compatible with the first signs of in vivo NK cell activation, as defined by the production of IFNγ, after microbial infection (21). Since DCs can efficiently prime NK cell responses (11–14) and since this function is more efficient if DCs are infected with bacteria (16), we hypothesized that, at least in the mouse system, IL-2 may be a key regulator of DC-dependent NK cell activation upon bacterial challenge. Our studies investigated the ability of IL-2 secreted by bacterially activated DCs to regulate NK cell functions.

Materials and Methods

Mice and Reagents. C57BL/6 and RAG2$^{-/-}$BALB/c mice were purchased from Harlan Italy. RAG2$^{-/-}$C57BL/6 mice were from Centre de Distribution, de Typage et d'Archivage Animal. IL-2$^{-/-}$C57BL/6 mice were provided by A. Schimpl (University of Würzburg, Würzburg, Germany). All animals were housed under pathogen-free conditions. For FACS® analysis and cell purifications, mAbs were purchased from BD Biosciences. F(ab′)$_2$ fragments were digested, as described (22), from ascite-purified anti–IL-2 (S4B6, rat IgG2a) or from total rat IgG (Bethyl). The IFNγ Duo Elisa kit (R&D Systems) was used to measure IFNγ in coculture supernatants. Polyclonal antibodies to mouse IFNα and IFNβ used to neutralize the bioactivity of type I IFNs were purchased from PBL Biomedical Laboratories. Normal rabbit serum was provided by the Animal Care Unit at the University of Western Australia.

BMDCs Preparation. BM cells from C57BL/6 or IL-2$^{-/-}$ C57BL/6 mice were cultured in IMDM (Euroclone) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol (all from Sigma-Aldrich), 10% heat-inactivated FBS (IMDM complete medium), and 10% supernatant of GM-CSF–transduced B16 tumor cells (23). Fresh medium was added every 2 d. After 7–10 d of culture, cells were analyzed for CD11c expression and used in assays when >90% were CD11c positive.

NK Cell Purification. NK cells were enriched by adhesion from spleens of RAG2$^{-/-}$ or purified from wild-type mice. For the enrichment protocol, single cell suspensions were plated in 100-mm tissue culture plates in IMDM complete medium and incubated for 1 h. Nonadherent cells (>40% DX-5$^+$ cells) were then recovered and used in appropriate assays. For purification from wild-type mice, NK cells were positively selected from splenocytes. 10^8 cells were stained with biotinylated anti–pan-NK cell (DX5) antibody (20 μg/ml) and washed and incubated with streptavidin MicroBeads (Miltenyi Biotech). Cells were then positively selected with MS columns, according to the manufacturer's recommendations. NK cells were used when >95% were NK1.1 positive.

NK–DC Co-cultures. Co-culture experiments were performed with NK cells from both wild-type and RAG2$^{-/-}$ mice. wtBMDCs or IL-2$^{-/-}$BMDCs were resuspended in IMDM complete medium without antibiotics and plated in 24-well plates (2.5×10^5 cells/well). Cells were treated with *Escherichia coli* DH5α at a multiplicity of infection (MOI) of 10 for 1.5 h, washed twice with PBS, and supplemented with IMDM complete medium containing 10% GM-CSF supernatant, 50 μg/ml gentamycin (Sigma-Aldrich), and 30 μg/ml tetracycline (Sigma-Aldrich). In some cases, activated BMDCs were cultured with rIL-2 (3 ng/ml), ascite-purified anti–IL-2, or rat IgG2a isotype control mAbs (5 μg/ml). After 0.5 h, NK cells (5×10^5 cells/well) were added directly to the culture or plated in a transwell insert, and 18 h later clarified supernatants were tested for IFNγ production. For some experiments IL-2$^{-/-}$BMDCs were plated in 24-well plates, activated or not with bacteria and after 5 h removed and fixed with glutaraldehyde as described (24). Fixed cells were resuspended in IMDM complete medium supplemented with 10% GM-CSF supernatant, gentamycin, tetracycline, and, where specified, rIL-2 (3 ng/ml).

In Vivo Activation of NK Cells. Mice were injected i.v. with 10×10^6 *E. coli* DH5α, and after 4 h spleens were removed and analyzed for NK cell activation. Single cell suspensions were prepared and incubated with brefeldin A (10 μg/ml; Sigma-Aldrich), ionomycin (100 ng/ml; Sigma-Aldrich), and phorbol 12-myristate 13-acetate (50 ng/ml; Sigma-Aldrich) for 3 h. Cells were fixed with 2% paraformaldehyde, permeabilized with PBS containing 5% FBS and 0.5% saponin, and stained with FITC-labeled anti-IFNγ and PE-labeled anti-NK1.1 (PK136) mAbs. Cells were then analyzed on a FACScan (Becton Dickinson). In some cases, mice were injected i.p. with F(ab′)$_2$ anti–IL-2 or rat IgG from day –3 until day 0 (1 mg/day) before bacterial challenge. Statistical analyses were performed using a two-tailed Student's *t* test.

RAG2$^{-/-}$ Mice Reconstitution. RAG2$^{-/-}$ mice were reconstituted with BMDCs by intraspleen injection of 5×10^6 wtBMDCs or IL-2$^{-/-}$BMDC. After 1 h, mice were injected with 10^7 bacteria. To test NK cell activation in vivo, the percentage of IFNγ-positive cells has been evaluated as described previously.

Depletion of T Cells. T cells were depleted by i.p. injection of purified anti-Thy antibody (T24-31.7) 2 d before bacterial infection. T cell depletion was confirmed by FACS® analysis of samples from blood and, after euthanasia, from spleens and lymph nodes.

E. coli Clearance. To test the efficiency of bacterial clearance, spleens were collected 2 h after *E. coli* injection, and unicellular suspensions were made in 3 ml of PBS. Pellets of single cell suspensions or centrifuged PBS supernatants were then plated on LB agar (Sigma-Aldrich), and colony-forming units were evaluated 24 h later.

B16 Melanoma Challenge. wtBMDCs or IL-2$^{-/-}$BMDCs were activated with *E. coli* DH5α at a MOI of 10 for 1 h, washed twice with PBS, and incubated 1 h in fresh IMDM complete medium supplemented with 10% GM-CSF supernatant, gentamycin, and tetracycline. BMDCs were then recovered, washed, and resuspended in PBS. RAG2$^{-/-}$ mice were injected i.v. with 2×10^6-activated wtBMDCs or IL-2$^{-/-}$BMDCs and soon after with B16 melanoma cells (2×10^5). After 14 d, lungs were removed and surface metastases were counted. Statistical analyses were performed using a two-tailed Student's *t* test.

Chromium Release Assay for NK Cell Cytotoxicity. NK cells recovered from DC-NK cocultures were counted to adjust for viable numbers of NK cells. NK cell cytotoxicity was determined by standard ^{51}Cr release assay. Briefly, viable NK cells were titrated twofold on 96-well plates, and ^{51}Cr-labeled YAC target cells (2×10^3) were added. Each assay was performed in triplicate. After a 4-h incubation at 37°C in 5%CO_2, ^{51}Cr release was measured as described (25). Data are presented as percentage of specific lysis calculated by the formula: percentage of specific lysis = (experimental cpm − spontaneous release cpm)/(total cpm −

spontaneous release cpm) × 100. Statistical analyses were performed using a two-tailed Student's *t* test.

Measurement of Type I IFN Activity. The biological activity of IFN-α/β was assessed using standard viral protection assays performed on vesicular stomatitis virus (VSV)-infected L929 fibroblast cultures as described (26).

Results

DC-derived IL-2 Is Required In Vitro to Elicit IFNγ Production from NK Cells. The ability of wild-type and IL-2–deficient bone marrow–derived DCs (wtBMDCs, IL-$2^{-/-}$ BMDCs) to induce IFNγ production by syngeneic NK cells was investigated. wtBMDCs and IL-$2^{-/-}$BMDCs were activated with *E. coli* at a MOI of 10, and after 2 h syngeneic NK cells were added to the DCs. Supernatants were collected 18 h later, and IFNγ production was measured. wtBMDCs were able to induce IFNγ production by syngeneic NK cells (Fig. 1 A), although this function was severely impaired in IL$2^{-/-}$BMDCs (Fig. 1 A). To verify that IFNγ was produced by NK cells rather than DCs, the presence of IFNγ-positive NK cells was confirmed by intracellular staining of $DX5^+$ cells after 4 h of coculture (Fig. 1 A). The inability of IL$2^{-/-}$BMDCs to activate NK cells was not due to a nonspecific lack of DC function as both wtBMDCs and IL$2^{-/-}$BMDCs were equally able to interact with NK cells and induce the up-regulation of the early activation marker CD69 (unpublished data). In IL$2^{-/-}$BMDCs, defects other than the ability to produce IL-2 after bacterial stimulation were excluded. wtBMDCs and IL$2^{-/-}$BMDCs were equivalently activated by bacterial stimulation (as measured by up-regulation of costimulatory and MHC molecules) and did not show any difference in viability (1) and in the production of various cytokines, including TNFα, IL-1β, IL-6, IL-10, IL-12, and IL-18 (unpublished data). We then analyzed whether DC-derived IL-2 was also required in an allogeneic context. Thus, the same coculture experiments were repeated, and BALB/c NK cells were incubated with C57BL/6 BMDCs. Again, a deficiency in IL-2 production by DCs resulted in a lack of NK cell activation (Fig. 1 B). A similar defect in the capacity of DCs to induce IFNγ production by NK cells was also observed when the effects of the IL-2 secreted by bacterially activated wtBMDCs were inhibited with a blocking anti–IL-2 antibody (Fig. 1 B). Importantly, exogenous rIL-2 added to DC-NK cocultures restored the capacity of IL$2^{-/-}$BMDCs to induce IFNγ secretion by both allogeneic and syngeneic NK cells (Fig. 1, A and B). These observations indicate that, at least in vitro, IL-2 derived from bacterially activated DCs is necessary for DC-mediated NK cell activation, as measured by their ability to produce IFNγ. We could exclude the role of other DC-derived cytokines, such as IL-12 and IL-18, in NK cell activation since bacterially activated IL-12– and IL-18–deficient BMDCs were as efficient as wtBMDCs in inducing IFNγ production by NK cells (unpublished data). Nevertheless, IL-2 is not the only factor required for this process. When bacterially activated DCs and NK cells were cultured separately in a transwell system, no IFNγ secretion by NK cells was observed (Fig. 1 C), indicating that soluble factors alone are not sufficient to trigger NK cell activation. Furthermore, exogenous rIL-2 added to unstimulated wtBMDCs did not render DCs capable of activating NK cells (Fig. 1 C). These data suggest that close cell to cell contact between NK cells and activated DCs is required, together with IL-2, for IFNγ release by NK cells.

DC-derived IL-2 Directly Activates NK Cells. Two possibilities exist to account for the role of DC-derived IL-2 in NK cell activation: either IL-2 directly activates NK cells or it exerts an autocrine activity on DCs, which in turn induces NK cells to secrete IFNγ. To distinguish between these two possibilities, we performed the following experi-

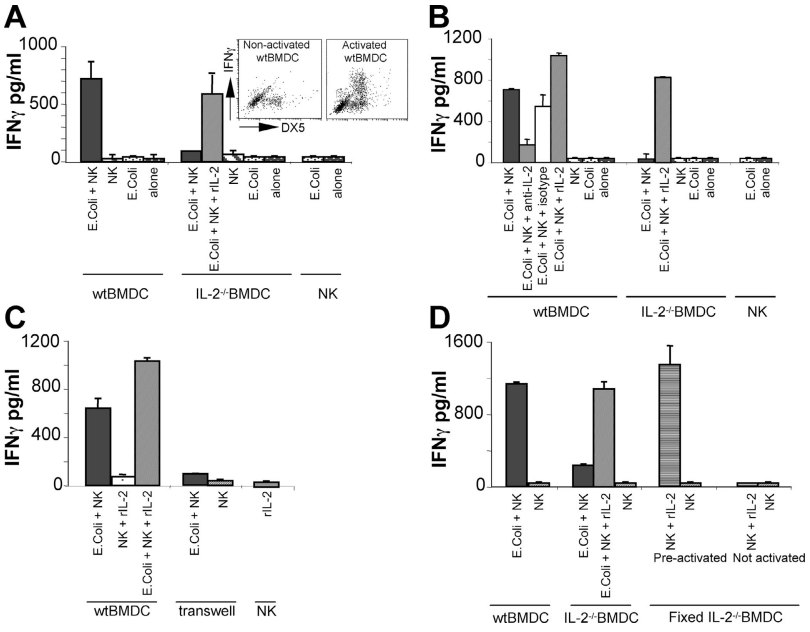

Figure 1. DC-derived IL-2 is a key molecule for DC-mediated NK cell activation in vitro. Immature or *E. coli*–activated wtBMDCs and IL-$2^{-/-}$BMDCs were cultured together with either (A) syngeneic or (B) allogeneic NK cells for 18 h. Levels of IFNγ in the supernatant were then quantified by ELISA. The insets in A represent the intracellular staining performed in the mixed DC-NK populations after 4 h of coculture. NK cells were identified as DX5 positive. In B, IL-2 was also blocked using the S4B6 anti–IL-2 antibody. (C) Cell contact–dependent activation of NK cells by wtBMDCs. Unstimulated or *E. coli*–activated wtBMDCs and allogeneic NK cells were cocultured in the same wells or separated by a porous membrane. NK cells alone were also cultured in the presence of rIL-2. IFNγ in the supernatant was measured by ELISA after 18 h of coculture. The experiments were repeated four times with similar results (A, B, and C). (D) IL-2 produced by activated DCs directly activates NK cells. Unstimulated or 5-h bacterially activated IL-$2^{-/-}$BMDCs were fixed and incubated with allogeneic NK cells. 18 h later, IFNγ was measured in the supernatant by ELISA. The experiment was repeated twice with similar results.

ment. IL2$^{-/-}$BMDCs were activated by interaction with bacteria and after 5 h were fixed. rIL-2 was then added to the fixed IL2$^{-/-}$BMDCs together with unprimed NK cells. IFNγ production by NK cells was tested after 18 h. Since the IL2$^{-/-}$BMDCs were fixed, the activity of rIL-2 was restricted to an effect exerted directly on NK cells. As shown in Fig. 1 D, activated and fixed IL2$^{-/-}$BMDCs induced IFNγ production from NK cells when supplied with exogenous rIL-2. In contrast, unstimulated and fixed IL2$^{-/-}$ BMDCs cultured in the presence of rIL-2 and activated and fixed IL2$^{-/-}$BMDCs not supplied with rIL-2 did not induce IFNγ release by NK cells. Production of IFNγ by NK cells was also observed after the addition of supernatant from bacterially activated wtBMDCs to activated and then fixed IL2$^{-/-}$BMDCs (unpublished data). These data indicate that IL-2 produced by bacterially activated DCs does not exert an autocrine effect on DCs but rather acts on NK cells and stimulates the secretion of IFNγ.

IL-2 Is Required In Vivo to Elicit IFNγ Production from NK Cells. If IL-2 produced by DCs after microbial exposure is necessary to induce IFNγ production by NK cells, IL-2–deficient mice should show a defect in NK cell activation in vivo early after bacterial infection. To test this hypothesis, IL-2–deficient mice were injected i.v. with *E. coli*, and NK cell activation was analyzed 4 h after infection by measuring the percentage of IFNγ-positive NK cells in the spleen. As expected, IL-2–deficient mice infected with bacteria were unable to mount an early NK cell response (Fig. 2 A). Similarly, a significant reduction ($P < 0.05$) in the efficiency of NK cell activation in vivo was observed by treating wild-type mice with a blocking anti–IL-2 antibody before bacterial challenge (Fig. 2 A). These results demonstrate that in vivo IL-2 is required for effective NK cell activation after bacterial infection.

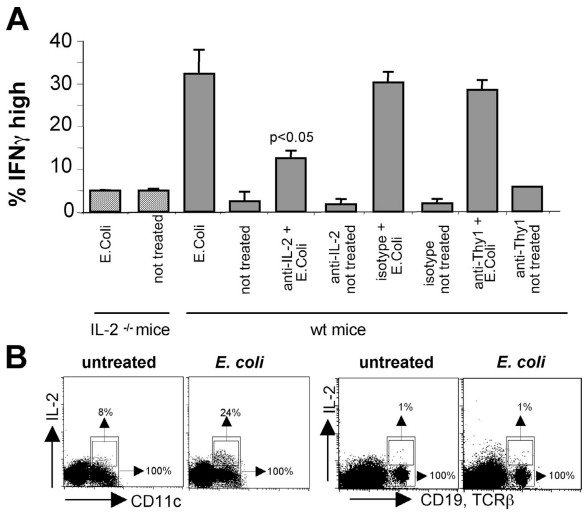

Figure 2. IL-2 is required in vivo for NK cell activation early after bacterial infection. (A) Frequency of IFNγ–producing NK cells in the spleen of untreated or *E. coli*–injected IL-2–deficient or wild-type mice measured by intracellular cytokine staining. The percentage of IFNγ-producing NK cells was also determined in wild-type mice pretreated with anti–IL-2, anti-Thy1, or isotype control antibodies before bacterial infection. The experiment was repeated three times with similar results. (B) Triple staining, 3 h after bacterial infection, of spleen cells with FITC-conjugated anti-CD11c antibody, PE-conjugated anti–IL-2 antibody, and biotinylated anti-CD19 and anti-TCRβ antibodies. In the left panels, the indicated fractions of IL-2–positive DCs have been calculated as the percentage of CD11c-positive cells (100%) before and after bacterial infection. Analogously, in right the panels the fraction of IL-2–positive non-DC leukocytes has been calculated as the percentage of CD19- or TCRβ-positive cells (100%) before and after bacterial challenge.

IL-2 Required In Vivo to Elicit IFNγ Production from NK Cells Does Not Have a T Cell Origin. To exclude the possibility that the IL-2 required for NK cell activation had a T cell origin, T lymphocytes were eliminated in wtC57BL/6 mice before bacterial challenge as described in Material and Methods. The efficiency of splenic NK cell activation 4 h after bacterial infection was then measured. As shown in Fig. 2 A, at early time points IFNγ production by NK cells after bacterial infection was independent from the presence of T cells.

We then performed an intracellular staining of spleen cells to verify if DCs were the principal population producing IL-2 early after microbial challenge. T and B lymphocytes were recognized using antibodies specific for CD19 and TCRβ, whereas DCs were identified using an anti-CD11c antibody. Thus, spleen cells were triple stained with FITC-conjugated anti-CD11c antibody, PE-conjugated anti–IL-2 antibody, and biotinylated anti-CD19 and anti-TCRβ antibodies. As shown in Fig. 2 B, the primary source of IL-2 produced very early after bacterial injection were CD11c-positive DCs. Together these results strongly suggest that the IL-2 required in vivo for efficient NK cell activation is primarily produced by DCs.

DC-derived IL-2 Is Required In Vivo to Elicit IFNγ Production from NK Cells. To investigate if IL-2 produced by DCs was required in vivo to elicit IFNγ production by NK cells, we took advantage of RAG2$^{-/-}$ mice. These animals, other than lacking T and B cells, which could represent other sources of IL-2, have been shown to develop nonfunctional DCs (27). We reasoned that if after bacterial infection in vivo the induction of IFNγ production by NK cell depends on DC activation, these mice should show a reduced efficiency in this process. As depicted in Fig. 3 A, in the absence of functional DCs the percentage of IFNγ-positive NK cells was indeed strongly reduced after bacterial challenge. Therefore, since RAG2$^{-/-}$ mice do not have endogenous DCs that can be activated, we were able to use them as recipients for IL-2–deficient or wtBMDCs to compare the effectiveness of these cells in inducing NK cell activation in vivo after bacterial challenge. RAG2$^{-/-}$ mice were injected in the spleen with wtBMDCs or IL2$^{-/-}$ BMDCs before bacterial infection, and the percentage of IFNγ-positive splenic NK cells was enumerated. As shown in Fig. 3 B, the efficiency of NK cell activation in RAG2$^{-/-}$ mice reconstituted with wtBMDCs was similar to that observed in wild-type mice. In contrast, RAG2$^{-/-}$ mice reconstituted with IL2$^{-/-}$BMDCs showed only a marginal increase in the percentage of IFNγ-positive NK cells (Fig.

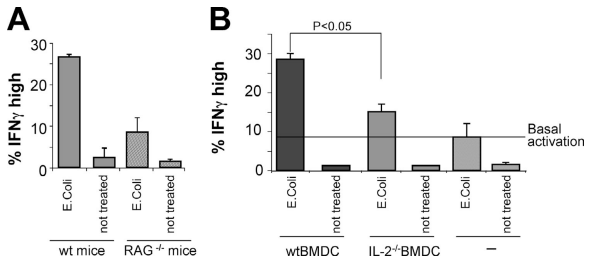

Figure 3. IL-2 produced by DCs is required in vivo for NK cell activation. (A) Frequency of IFNγ-producing NK cells in the spleen of untreated or *E. coli*–injected RAG2$^{-/-}$ or wild-type mice. The experiment was repeated three times with similar results. (B) Frequency of IFNγ-positive NK cells in the spleen of RAG2$^{-/-}$ mice injected intraspleen with wtBMDCs, IL-2$^{-/-}$BMDCs or not injected (-), and then treated or not with 10^7 *E. coli* i.v. Data are means and SDs from four mice analyzed in two independent experiments.

3 B) in comparison to basal percentages (i.e., percentages of IFNγ-positive NK cells in RAG2$^{-/-}$ mice infected with *E. coli* only). Together these results show that the presence of functional DCs capable of producing IL-2 is required in vivo to efficiently induce IFNγ production by NK cells.

DC-derived IL-2 Is Required to Elicit Antibacterial and Antitumor NK Cell Responses In Vivo. The functional relevance of DC-derived IL-2 in activation of NK cell antibacterial and antitumor responses was then investigated. The role of IFNγ in enhancing the phagocytic activity of macrophages and other phagocytic cells is well characterized (16, 17). Thus, we investigated whether the clearance of i.v.-injected bacteria was different in RAG2$^{-/-}$ mice reconstituted with wtBMDCs that strongly induce the production of IFNγ by NK cells or with IL2$^{-/-}$BMDCs that are inefficient in activating NK cells as previously described (Fig. 3 B). For this purpose, 10 million *E. coli* were injected i.v. in DC-treated RAG2$^{-/-}$ mice, and the bacterial titers in spleens were evaluated 2 h later. At early time points, the only source of IFNγ in vivo are NK cells as shown in Fig. 4 A. The number of bacteria in the spleens of mice treated with IL2$^{-/-}$BMDCs was significantly ($P < 0.05$) higher than that in spleens of mice treated with wtBMDCs (Fig. 4 B).

In the absence of NKT cells, development of lung metastases after i.v. administration of B16 melanoma cells can be prevented by activated NK cells (28–30) via the production of IFNγ (31). Here, we evaluated the level of protection against the formation of melanoma metastases exerted by NK cells primed with wild-type or IL-2–deficient DCs activated by bacterial encounter. B16 tumor cells were injected i.v. into RAG2$^{-/-}$ mice together with *E. coli*–activated wtBMDCs or IL2$^{-/-}$BMDCs, and lung metastases were enumerated after 2 wk. Treatment with bacterially activated wtBMDCs significantly ($P < 0.05$) reduced metastasis formation compared with treatment with IL2$^{-/-}$ BMDCs (Fig. 4 C). The size of the metastases was also very different in the two experimental conditions. All the metastases that developed in mice treated with IL2$^{-/-}$BMDCs were visible by naked eye, whereas the metastases that developed in mice treated with wtBMDCs were not visible and had to be counted using a dissecting microscope (unpublished data). These observations confirmed the functional relevance of IL-2 produced by bacterially activated DCs in the activation of NK cells. Our results indicate that DCs acquire the ability to trigger NK cell–mediated IFNγ responses soon after microbial challenge and that this process is mediated by DC-derived IL-2.

Efficient Acquisition of Lytic Function by NK Cells Is Regulated by Type I IFNs Produced by Bacterially Activated Myeloid DCs. Finally, we investigated whether bacterially activated DCs were also able to prime NK cell cytotoxicity. NK cells were cocultured with bacterially activated syngeneic DCs for 24–48 h, and their ability to kill MHC-negative targets was then measured. *E. coli*–activated DCs primed NK cell cytotoxicity, but unlike regulation of IFNγ production cytotoxicity was not dependent on IL-2. In fact, IL2$^{-/-}$BMDCs were as efficient as wtBMDCs in inducing NK cell–mediated lysis (Fig. 5 A). In addition, the activation of NK cell cytolysis was not dependent on DC-derived IL-12 and IL-18, since after bacterial infection BMDCs from IL-12– and IL-18–deficient mice induced NK cell cytotoxicity equivalent to that induced by wtBMDCs (unpublished data). NK cell cytotoxicity and cytokine production can be autonomously regulated in response to distinct cytokine-induced signaling pathways (32). In particular, during viral infections NK cell cytotoxicity requires STAT1 activation and is principally induced by type I IFNs (32). Since myeloid DCs can produce type I IFNs after viral challenge (33), we tested

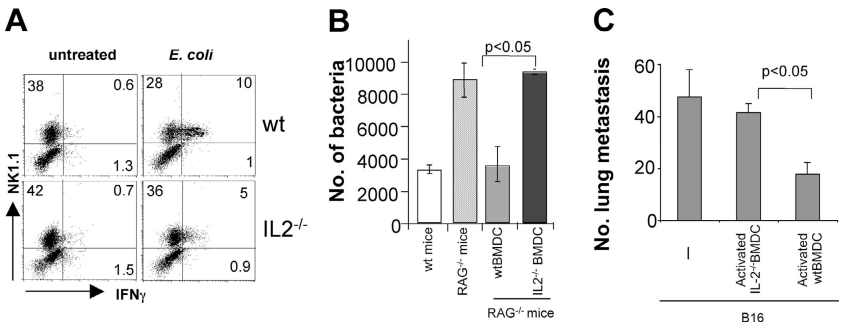

Figure 4. DC-derived IL-2 is required in vivo to elicit antibacterial and antitumor NK cell activity. (A) Double staining with anti-NK1.1 and anti-IFNγ antibodies of spleen cells from RAG2$^{-/-}$ mice injected (*E. coli*) or not (untreated) with 10^7 bacteria. Before bacterial injection, mice were reconstituted either with wtBMDCs (wt) or IL2$^{-/-}$BMDCs (IL2$^{-/-}$), and the intracellular staining was performed 2 h after bacterial treatment. Percentages of cells in each quadrant are indicated. (B) Titers of free bacteria 2 h after i.v. injection of 10^7 *E. coli* in the spleens of wild-type or RAG2$^{-/-}$ mice reconstituted with either wtBMDCs or IL2$^{-/-}$BMDCs. The experiment was repeated twice with similar results. (C) The number of lung metastases in mice 14 d after i.v. injection of B16 melanoma alone (−), B16 melanoma and bacterially activated IL-2$^{-/-}$BMDCs, or B16 melanoma and bacterially activated wtBMDCs. Data represent means and SDs from three independent experiments.

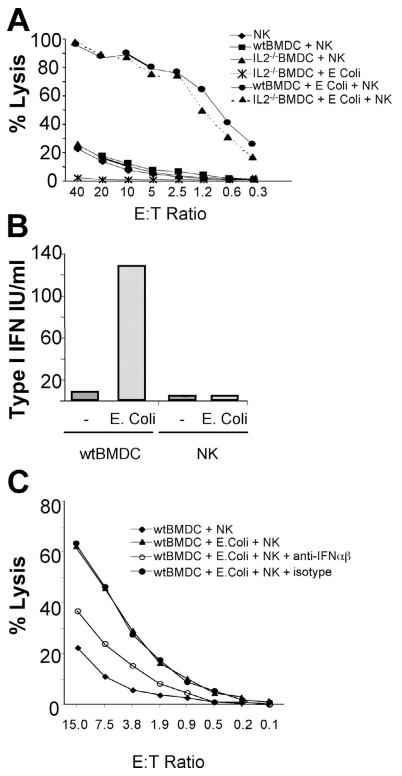

Figure 5. NK cell cytotoxicity is regulated by DC-derived type I IFNs. (A) DC-derived IL-2 is not required for the induction of NK cell cytotoxicity. Untreated or *E. coli*–activated wtBMDCs and IL-$2^{-/-}$BMDCs were cocultured with syngeneic NK cells for 48 h. NK cell cytotoxicity presented as the percentage of lysis of YAC targets was measured by a standard ^{51}Cr release assay. (B) Bacterially activated BMDCs secrete type I IFNs. The amount of IFNαβ in the supernatant of wtBMDCs and NK cell cultures was measured after exposure to *E. coli* by a standard viral protection assay. (C) Type I IFNs control the induction of NK cell cytotoxicity after bacterial infection. Untreated or *E. coli*–activated wtBMDCs were cocultured with syngeneic NK cells for 24 h in the presence or absence of IFNαβ-blocking antibodies ($P < 0.0001$ when the percentage of lysis is compared between wtBMDC + *E. coli* + NK + isotype and wtBMDC + *E. coli* + NK + anti-IFNαβ at an E:T ratio of 7.5:1). The data are representative of three independent experiments conducted with triplicate samples.

whether they could also acquire this ability in response to bacterial encounter. As shown in Fig. 5 B, wtBMDCs produced type I IFNs upon bacterial challenge. Thus, we investigated the relevance of these molecules in inducing NK cell cytotoxicity. Neutralizing the activity of type I IFNs in DC-NK cocultures resulted in a significant reduction in NK cell cytotoxicity (Fig. 5 C) ($P < 0.0001$), suggesting that the efficient acquisition of lytic function by NK cells was regulated by type I IFNs produced by bacterially activated DCs.

Discussion

The cross talk between DCs and NK cells has been described in the context of immune responses to infectious agents and tumors (13, 14, 16, 34). The molecular basis of these interactions, however, remained largely unrevealed. Here, we defined some of the DC cytokines that control the effector functions of NK cells. We showed that IL-2 produced early by bacterially activated mouse DCs plays a fundamental role in the activation of NK cell–mediated immunity in vitro and in vivo. This indicates that besides its well-defined function in acquired immunity, IL-2 is also necessary, at least after bacterial infections, for the regulation of innate immune responses.

Bacteria and bacterial cell products but not inflammatory cytokines can induce DCs to produce IL-2 (20). Nevertheless, it has been shown that inflammatory cytokines, such as TNFα, may render DCs able to activate NK cells (14). Therefore, it is possible that in response to diverse physiological stimuli factors other than IL-2 are involved in DC-NK cross talk.

The specific contribution of IL-2 to the efficient activation of NK cells described in the present work has been studied in the mouse system. Our recent studies have shown that human DCs are also capable of producing IL-2 in an IL-15–dependent manner (unpublished data). Thus, it remains to be investigated if also in humans IL-2 could play a role in stimulating NK cells.

IL-12 and IL-18 have been uncontroversially shown to be involved in NK cell activation (35). Nevertheless, at least in vitro, we excluded a role of these cytokines in DC–NK interactions after bacterial encounter, since IL-12– and IL-18–deficient BMDCs were as efficient as wt-BMDCs in inducing NK cell activation (unpublished data). This observation extends previous studies performed in mouse and human systems in which DC-mediated activation of NK cells was shown to be independent of IL-12, IL-18, and also IL-15 (18, 36). In these systems, activation of NK cells by DCs required cell to cell contact, and separation of DC and NK populations in transwells inhibited NK cell activation. In our system, bacterially activated DCs need to produce IL-2 but also need to establish cell to cell contact with NK cells for IFNγ release by NK cells to occur. Thus, IL-2, though necessary, is not sufficient to induce NK cell activation, and additional interactions are required. These interactions could be necessary either to concentrate IL-2 locally at the contact site or to allow the recognition between some surface molecules. The use of blocking reagents against numerous surface receptors, such as CD80, CD86, CD154, CD11a, CD50, and Nkp30 failed to reveal a role for these membrane-associated determinants in the productive interaction between DCs and NK cells (12, 16). Here, we tested the possible relevance of other surface molecules, and we excluded the involvement of NKG2D, ICOSL, and CX3CR1 (unpublished data) in NK cell activation mediated by bacterially activated DCs.

Since DCs express functional IL-2 receptors and up-regulate the IL-2Rα subunit after activation, the possibility existed that IL-2 exerted its function on DCs in an autocrine manner by contributing to the DC maturation process. This hypothesis was dismissed by our findings that IL-2 is directly required for NK cell activation. Moreover, no clear IL-2 effect on DC maturation measured by up-regulation of costimulatory or MHC molecules and acquisition

of T cell priming capacity was observed in our studies (unpublished data).

Residual DC-dependent NK cell activity can be induced in vivo in the absence of IL-2, a phenomenon not observed in our in vitro assays. Previous in vitro studies have shown that DCs may acquire the capacity to induce NK cell–mediated IFNγ secretion independently of microbial infection and IL-2 production (13) provided they are exposed to IL-4 (14, 37). It is possible that the residual NK activation observed in vivo in the absence of IL-2 is due to an alternative mechanism of DC conditioning, possibly involving IL-4. Moreover, we cannot exclude the possibility that in bacterial infections cytokines such as IL-15 may represent additional factors required for DC-dependent NK cell activation in vivo. Since bacterially activated DCs produce type I IFNs, a role for IL-15 is particularly attractive as this cytokine has been shown to be produced by DCs in response to type I IFNs (38).

The involvement of NK cells in antitumor responses has been observed in different experimental systems (39). Moreover, in patients with cancer NK cell activities have been shown to be impaired as assessed by the reduced functionality of NK cells from patients ex vivo. In agreement with these observations, apparently disease-free patients with functional peripheral blood NK cells have a significantly longer metastasis-free survival time than those with low NK cell activity (15). Antitumor NK cell functionality is strongly increased after activation (39). The ability of bacterially primed DCs to induce antitumor NK cell activities may explain the efficacy of bacterially based immune therapies (40, 41), and studies presented in this paper provide important clues as to how DCs may prime NK cell–mediated antitumor functions. Indeed, in our mouse system the antimetastatic effects of NK cells are dependent on the IL-2 released by bacterially activated DCs. Independent studies analyzing two different tumor models in vivo reported that NK cell activation is independent of IL-12 and IL-15 (18, 36). At least for IL-12, the reported observations are in agreement with our in vitro experiments in which DC-mediated activation of NK cell cytotoxic and secretory functions did not depend on this cytokine.

Bacterially activated DCs elicit NK cell cytotoxicity in an IL-2–independent manner. This is somehow surprising since NK cells exposed for long periods to high doses of soluble IL-2 acquire both secretory and cytotoxic activities. However, NK cell cytotoxicity and cytokine production are independently activated and involve distinct cytokine-induced signaling pathways (32). Although activation of STAT4 is critical for IFNγ production by NK cells, the acquisition of cytotoxic function requires STAT1 signaling (32). Although IL-2 is able to activate the STAT1, STAT4, and STAT5 signaling pathways in NK cells in vitro (42), IL-2–mediated STAT1 activation is inefficient (43). Usually, the IL-2 doses to which NK cells are exposed in vitro to induce activation are extremely high and possibly sufficient to efficiently activate the STAT1 pathway and thus induce NK cell cytotoxicity. In contrast, the IL-2 amounts to which NK cells are exposed in the cocultures with bacterially activated DCs are low and thus presumably not sufficient to induce effective activation of the STAT1 pathway.

Activation of STAT1 in NK cells has been described to be predominantly mediated by type I IFNs (32). It has been shown recently that type I IFNs can be produced not only by plasmacytoid DCs but also by myeloid DCs exposed to viruses or viral products (33). In this study, we found that myeloid DCs derived in vitro from BM precursors can produce type I IFNs after bacterial activation. The precise contribution of myeloid and plasmacytoid DCs in the production of type I IFNs in different physiological circumstances in vivo remains to be defined. Our results suggest that production of type I IFNs may represent a poorly recognized response of myeloid DCs to several infectious agents, including bacteria.

The up-regulation of NK cell cytotoxicity by bacterially activated DCs may not be directly required for their antibacterial effects. The biological relevance of this response may relate to the fact that cytotoxicity could contribute to controlling the late phases of the immune response by limiting inflammation and restoring homeostatic balance after infection (16, 37). DCs are susceptible to NK cell–mediated lysis, hence the ability to elicit NK cell cytotoxicity may be a means for DCs to limit their own activity. Furthermore, we would like to propose that, given the ability of NK cells to acquire strong cytolytic function after interaction with bacterially activated DCs, bacterial infections may contribute to maintaining a basal level of alert against tumors.

Our findings reveal novel aspects of the molecular mechanisms that contribute to DC–NK interactions both in vitro and in vivo and define an new role for IL-2 in innate immunity. Understanding the regulation of innate immune responses and defining the critical mediators places us closer to effectively manipulate these responses to improve therapeutic outcomes.

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