TLR-Dependent Activation Stimuli Associated with Th1 Responses Confer NK Cell Stimulatory Capacity to Mouse Dendritic Cells

Ivan Zanoni, Maria Foti, Paola Ricciardi-Castagnoli and Francesca Granucci

*J Immunol* 2005; 175:286-292; doi: 10.4049/jimmunol.175.1.286

http://www.jimmunol.org/content/175/1/286

**References**

This article cites 48 articles, 24 of which you can access for free at: http://www.jimmunol.org/content/175/1/286.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
TLR-Dependent Activation Stimuli Associated with Th1 Responses Confer NK Cell Stimulatory Capacity to Mouse Dendritic Cells

Ivan Zanoni, Maria Foti, Paola Ricciardi-Castagnoli, and Francesca Granucci

Dendritic cells (DCs) have an important role in the activation of NK cells that exert direct antitumor and antimicrobial effects and can influence the development of adaptive T cell responses. DCs acquire NK cell stimulatory capacity after exposure to various stimuli. In this study we investigated the nature of the stimuli that confer to DCs the NK cell-activating capacity. After exposure of DCs to TLR-dependent and -independent microbial stimuli and to nonmicrobial stimuli, we evaluated the ability of activated DCs to elicit IFN-γ production from NK cells in vitro and to promote NK cell activation in vivo. We show in this study that only TLR-dependent microbial stimuli typically associated with Th1 responses confer to DCs the ability to activate NK cells, whereas stimuli associated with Th2 responses do not have this property. The Journal of Immunology, 2005, 175: 286–292.

Mice and reagents
C57BL/6 and BALB/c mice were purchased from Harlan Italy. All animals were housed under pathogen-free conditions. For FACS analysis and cell purifications, mAbs were purchased from BD Pharmingen. The IFN-γ and IL-2 Duo Elisa kit (R&D Systems) were used to measure IFN-γ and IL-2 production from NK cells in vitro. Quantikine immunoassays (R&D Systems) for CXCL9 and CXCL10 were used to quantify chemokine production by DCs. The stimuli used were Escherichia coli LPS (Sigma-Aldrich; 10 μg/ml), CpG oligonucleotides (Primm; 1 μM), Pam3Cys (EMC Microcollections; 10 μg/ml), endotoxin free (endotoxin concentration in the stock solution, <0.0007 ng/ml according to the Limulus test) CT (List Biological Laboratory; 1 μg/ml), PGE2 (Sigma-Aldrich; 1 μM), bacillus Calmette-Guérin (BCG; Sigma-Aldrich; multiplicity of infection (MOI), 10), Leishmania mexicana promastigote (Sigma-Aldrich; MOI, 5), and PT (Sigma-Aldrich; 1 μg/ml). Before use, PT was purified on endotoxin removal columns (Detoxi-Gel; Pierce).
The studies were reviewed and approved by an appropriate institutional review committee.

**BMDC preparation**

Bone marrow cells from C57BL/6 or BALB/c mice were cultured in IMDM (Euroclone) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME (all from Sigma-Aldrich), 10% heat-inactivated FBS (IMDM complete medium), and 10% supernatant of GM-CSF-transduced B16 tumor cells (25). Fresh medium was added every 3 days. After 7–10 days of culture, cells were analyzed for CD11c expression and were used in assays when >90% were CD11c positive.

**NK cell purification**

NK cells were positively selected from splenocytes of BALB/c and C57BL/6 mice. Cells (10⁶) were stained with biotinylated anti-pan-NK cell (DX5) Ab (20 μg/ml), washed, and incubated with streptavidin MicroBeads (Miltenyi Biotec). Cells were then positively selected with Mini-MACS separation columns (Miltenyi Biotec), according to the manufacturer’s recommendations. NK cells were used when >90% were DX5 positive.

**Splenic DC purification**

DCs were obtained from BALB/c and C57BL/6 mice by negatively selecting CD19⁺, CD3⁺, panVβ⁺, F4/80⁺, DX5⁺, and GR1⁺ cells using the MicroBeads (Miltenyi Biotec) system. The resulting population was >80% CD11c⁺ and did not show any T or B cell contamination (data not shown).

**DC-NK cell cocultures**

DCs were resuspended in complete IMDM without antibiotics, plated in 48-well plates (1.25 × 10⁵ cells/well), and treated with various stimuli. In the experiments performed with microorganisms, DCs were treated with BCG or L. mexicana, respectively, at MOI of 10 and 5 for 1.5 h, washed twice with PBS, and supplemented with complete IMDM containing 10% GM-CSF, gentamycin (50 μg/ml; Sigma-Aldrich), and tetracyclin (30 μg/ml; Sigma-Aldrich). In some cases, activated DCs were cultured with rIL-2 (3 ng/ml), ascite-purified anti-IL-2 (S4B6 clone), or rat IgG2a isotype control mAbs (5 μg/ml). After 0.5 h, NK cells (2.5 × 10⁵ cells/well) were added directly to the culture; 18 h later, clarified supernatants were tested for IFN-γ production. For the intracellular staining, cells were incubated with brefeldin A (10 μg/ml; Sigma-Aldrich) for 4 h after 4 h of coculture. Cells were fixed with 2% paraformaldehyde, permeabilized with PBS containing 5% FBS and 0.5% saponin, and stained with anti-IFN-γ, anti-CD11c and anti-DX5 (BAlb/c), or anti-NK1.1 (C57BL/6) mAbs.

**IL-2 production by activated DCs**

IL-2 was measured by ELISA in the supernatants of 18-h activated DCs. For the intracellular staining, DCs were incubated with brefeldin A (10 μg/ml; Sigma-Aldrich) for 4 h after 2 h of activation. Cells were fixed with 2% paraformaldehyde, permeabilized with PBS containing 5% FBS and 0.5% saponin, and stained with anti-IL-2 and anti-CD11c mAbs.

**In vivo NK cell recruitment and activation**

Immature DCs and DCs (10⁶) exposed for 2 h in vitro to various activation stimuli were injected s.c. at the tail base. The number of NK cells in the draining inguinal lymph nodes was enumerated 24 h later by FACS analysis. For intracellular staining, single cell suspensions of draining lymph nodes were prepared and incubated with brefeldin A (10 μg/ml; Sigma-Aldrich), ionomycin (100 ng/ml; Sigma-Aldrich), and PMA (50 ng/ml; Sigma-Aldrich) for 4 h. Cells were fixed with 2% paraformaldehyde, permeabilized with PBS containing 5% FBS and 0.5% saponin, and stained with FITC-labeled anti-IFN-γ mAb. In BALB/c mice, NK cells were identified by staining with DX5 and anti-asialo-GM1 Abs; in C57BL/6 mice, NK cells were identified as NK1.1⁺CD5⁺ cells.

**Results**

Only DCs activated with TLR-dependent microbial stimuli are able to induce NK cells to produce IFN-γ

The process of DC maturation depends on the nature of the stimulus. Indeed, DCs show diverse responses to different stimuli and this might influence the outcome of the immune reaction. To investigate the stimuli that could render DCs capable of activating NK cells, BALB/c and C57BL/6 bone marrow-derived DCs activated with diverse full-maturation stimuli were cocultured with syngeneic NK cells. The ability of activated DCs to prime NK cells was verified by testing the capacity of NK cells to produce IFN-γ. Coculture supernatants were collected 18 h later, and IFN-γ production by NK cells was measured. In this experiment we used full maturation, TLR-dependent and -independent microbial stimuli and full-maturation, nonmicrobial stimuli. The TLR-dependent microbial stimuli used were: LPS (TLR4), CpG (TLR9), Pam3Cys (TLR2) (26), BCG, and L. mexicana promastigote (27, 28). The TLR-independent microbial stimuli were CT and PT. The nonmicrobial inflammatory stimuli we used was PGE₂.

As shown in Fig. 1, only DCs activated by TLR-dependent microbial stimuli were able to induce IFN-γ production by NK cells, whereas DCs exposed to TLR-independent microbial stimuli or to inflammatory nonmicrobial stimuli could not elicit IFN-γ production from NK cells. The only exception was Pam3Cys, which conferred to DCs the ability to activate NK cells only in the context of

**FIGURE 1.** IFN-γ production by NK cells cocultured with DCs stimulated with various activation stimuli. Immature DCs or DCs activated with the indicated stimuli were cultured together with syngeneic NK cells for 18 h. Levels of IFN-γ in the supernatants were then quantified by ELISA. Left panel, DCs and NK cells on the BALB/c background; right panel, DCs and NK cells on the C57BL/6 background. DC⁺NK, DCs activated with the indicated stimuli cocultured with NK cells; DC, DCs stimulated with the indicated stimuli without NK cells; NK, NK cells stimulated with the indicated stimuli without DCs; iDC, immature DCs; iDC⁺NK, immature DCs cocultured with NK cells. The experiment was repeated three times with similar results. Insets represent the intracellular staining performed in the mixed DC-NK populations after 4 h of coculture. DCs were activated with the indicated stimuli. Cocultured cells were triple-stained with anti-IFN-γ, anti-CD11c, and anti-DX5 (for BALB/c cells) or anti-NK1.1 (for C57BL/6 cells) mAbs and analyzed by FACS. NT, nonactivated DCs cocultured with NK cells.
IL-2 derived from DCs activated with TLR-dependent microbial stimuli is required to elicit IFN-γ production from NK cells

We have previously shown that, in the case of E. coli infections, IL-2 produced early by bacterially activated mouse DCs is required for the activation of NK cell-mediated immunity in vitro and in vivo (7). Thus, we tested whether the stimuli used in the previous experiment could induce DCs to produce IL-2. The presence of IL-2 in the supernatants of activated DCs was measured 24 h after the stimulus encounter. In agreement with our previous observation (7, 29), we found that only DCs activated with TLR-dependent microbial stimuli were able to produce IL-2, whereas DCs activated with all other stimuli did not demonstrate this property (Fig. 2A). To verify that IL-2 was actually produced by DCs and not by bone marrow-contaminating cells, the presence of IL-2-positive, CD11c-positive cells was confirmed by intracellular staining 2 h after activation (Fig. 2A, lower panels).

The stimuli that could not induce IL-2 production by DCs could, however, induce activation, as shown by the up-regulation of activation markers (Fig. 2B). The results were similar for BALB/c and CB57BL/6 DCs as well as for DCs after incubation with NK cells (data not shown).

To test whether DC-derived IL-2 was required to elicit IFN-γ from NK cells, the effects of IL-2 secreted by activated DCs were blocked in DC-NK cell cocultures using a blocking anti-IL-2 Ab. As shown in Fig. 3A, blocking IL-2 completely inhibited the capacity of DCs to activate NK cells. Conversely, the addition of exogenous IL-2 to the DC-NK cell cocultures in which DCs were activated with molecules unable to stimulate IL-2 production was not sufficient to induce NK cell activation. The only exception was Pam3Cys in the C57BL/6 background, suggesting that the inability of C57BL/6 DCs activated with Pam3Cys to elicit IFN-γ production from NK cells was due to their inability to produce IL-2 in this context. Together, these data suggest that IL-2 is not the only factor required for IFN-γ production by NK cells in DC-NK cell interactions.

The requirement of DC-derived IL-2 for NK cell activation was confirmed in freshly purified splenic CD11c+ DCs. In this experiment CD11c+ cells were obtained from spleens by negative selection, activated with TLR-dependent microbial stimuli, and cocultured with syngeneic NK cells for 18 h in the presence of the absence of IL-2-blocking Ab. As shown in Fig. 3B, IFN-γ was produced by NK cells in an IL-2-dependent manner. The lower efficiency of NK cell activation observed under these conditions could be due to the fact that most of the ex vivo splenic DCs were already in a mature state and did not respond to additional stimulation (data not shown).

NK cell activation in vivo

It has been recently shown that during an inflammatory process, NK cells can be recruited at the lymph nodes, where they play a role in T cell priming. This process is regulated by IL-4 and LPS-stimulated DCs that, when injected s.c., migrate to the draining lymph nodes (9, 30) and recruit and activate NK cells (9, 31). NK cell activation in the lymph nodes can be tested by measuring the up-regulation of CD69 (9). We thus considered this phenomenon as a parameter to investigate in vivo the type of stimuli that could confer to DCs NK cell stimulatory capacity and involve them in the immune response. To perform this experiment, DCs activated with different stimuli were injected s.c., and, 24 h later, the number of NK cells in the draining lymph nodes was enumerated. We also evaluated the percentage of activated, CD69+, NK cells. As shown in Fig. 4, A and B, only TLR-dependent stimuli conferred to DCs NK cell stimulatory capacity in vivo. In agreement with this observation, DCs exposed to these activation stimuli were able to
induce IFN-γ production by NK cells, as indicated by the presence of IFN-γ-positive NK cells in the draining lymph nodes (Fig. 4C). As previously observed, Pam3Cys had an effect only in BALB/c, not in C57BL/6, mice (Fig. 4).

In contrast, concerning NK cell recruitment/proliferation, among the TLR-dependent stimuli, Pam3Cys did not show any effect in BALB/c or C57BL/6 mice, whereas LPS and CpG were very effective (Fig. 5A). Among the TLR-independent microbial stimuli, PT, although unable to confer to DCs the ability to activate NK cells, was efficient in making DCs capable of inducing recruitment/proliferation of NK cells at the draining lymph nodes (Fig. 5A).

It has been shown that NK cell recruitment at the draining lymph nodes is partially dependent on CXCR3 (31). Therefore, we evaluated whether the various activation stimuli could induce different CXCR3 ligand production by mouse DCs. To this purpose, CXCL9 and CXCL10 were measured in the supernatants of activated DCs 18 h after stimulation. Similar to the previous observation, LPS and CpG very efficiently induced the production of both chemokines by DCs, whereas Pam3Cys, CT, and PGE2 did not have any effect (Fig. 5B), suggesting that CXCR3L may be involved in NK cell recruitment. In contrast, PT, although able to induce NK cell accumulation, led to only a weak release of CXCL10 (Fig. 5B), suggesting that in this case, NK cell enrichment at the draining lymph nodes might be due to different mechanisms.

Discussion

We have shown in this study that TLR-dependent, and not TLR-independent, full-maturation stimuli confer to DCs the ability to elicit IFN-γ production by NK cells. Moreover, migrating DCs activated with specific full maturation stimuli are able to recruit or induce NK cell proliferation at the draining lymph nodes.

It has been recently observed that activated NK cells can provide in the lymph nodes an early source of IFN-γ necessary for subsequent polarization of T cell responses toward Th1 (31). The activation of the Th1 program depends on the activity of the transcription factor T-bet, which is induced by TCR and IFN-γR triggering (32). Consistent with this observation, the stimuli that we found able to confer to DCs NK cell stimulatory capacity were typically associated in vivo with Th1 responses. In particular, LPS and CpG were able to induce DC-mediated NK cell activation (IFN-γ production) and recruitment in vitro and in vivo, whereas the Th2 stimulus Pam3Cys, although it could stimulate DCs to
elicit IFN-γ production from NK cells in vitro in the BALB/c background, was not able to promote in vivo DC-mediated NK cell recruitment/proliferation at the draining lymph nodes. Because under steady state conditions, NK cells are present in very low numbers in mouse lymph nodes (9), their recruitment/proliferation and activation could be relevant to have enough IFN-γ for Th1 polarization.

PT has also been associated with Th1 responses observed during Bordetella pertussis infections (33). In agreement with this, we found that PT was able to induce DC-mediated NK cell activation capacity in vitro and in vivo. Because we observed that this activity is attributable only to TLR-dependent full-activation stimuli, we could hypothesize that in Bordetella infections, Bordetella microbial-associated molecular patterns are required for NK cell activation, and PT is necessary to optimize NK cell recruitment. It has been shown that NK cell recruitment at the draining lymph nodes is partially dependent on CXCR3 (31). In line with this observation, we have found that DCs activated with the microbial stimuli (LPS and CpG) able to guarantee NK cell accumulation produce large amounts of two CXCR3Ls, CXCL9 and CXCL10. This suggests that the accumulation of NK cells at the draining lymph nodes might be due to DC-mediated, CXCR3-dependent NK cell recruitment. Conversely, PT induces very low CXCL9 and CXCL10 expression; this indicates that in this case either other chemokines are involved in the NK cell recruitment process, or PT-matured DCs stimulate local NK cell proliferation.

TLR-dependent, full-activation stimuli can make DCs able to induce IFN-γ production by NK cells. This function is dependent on IL-2 secreted by activated DCs and other mediators produced in this context. In fact, blocking the effects of IL-2 in DC-NK cocultures in presence of TLR-dependent, full-activation stimuli resulted in a lack of NK cell priming, whereas the addition of exogenous IL-2 in DC-NK cocultures when DCs were activated with TLR-independent, full-activation stimuli was not sufficient to elicit IFN-γ secretion by NK cells. We have observed in E. coli-activated DCs (7) that ICOS ligand and CX3CR1 were not additional mediators. In agreement with a previous work, we can also exclude the effect of costimulatory molecules, such as B7.1, B7.2,
A representative experiment of three is shown. NT, untreated mice. BALB/c mice and with anti-NK1.1 and anti-CD3 Abs in C57BL/6 animals. Identified by double staining with anti-DX5 and anti-asialo-GM1 Abs in injection of DCs activated with the indicated stimuli. NK cells were identified on microbial stimuli (7, 9). IL-4 is, indeed, a DC semimature molecule that we observed with freshly isolated splenic DCs in the presence of blocking IL-2 Ab (Fig. 3B) could be due to previous exposure of some of the purified DCs to IL-4.

Consistent with the observation that the TLR-dependent, full-activation stimuli able to render DCs efficient stimulators of NK cells are stimuli associated with Th1 responses, L. mexicana-activated DCs could not elicit IFN-γ production from NK cells. Infections with L. mexicana have been associated with the development of Th2 responses in many mouse strains (39), in contrast to L. major infections that lead to the development of a protective Th1 response (40). In the context of L. major infections, the development of a Th1 response could be due to the activation of NK cells in lymph nodes. In fact, it has been shown that the IFN-γ required to activate protective CD4+ T cells in response to L. major is produced by NK cells (41).

Besides L. mexicana, the other Th2-associated, TLR-independent, inflammatory stimuli we used, CT and PGE2, could not confer to DCs the ability to activate and recruit NK cells at the draining lymph node.

The activation of NK cells is important during microbial (bacterial) infection not only to influence subsequent T cell responses, but also to provide a direct early antimicrobial effect exerted by IFN-γ that potently activates phagocytes (4, 42). This is consistent with the activity of the TLR-dependent activation stimuli described in this study.

Direct involvement of NK cells has been also demonstrated in antitumor responses in different experimental systems (43), and the role of NK has been unequivocally observed in patients with cancer (44). The ability of TLR-dependent microbial stimuli to render DCs capable of activating NK cells may explain the efficacy of bacterially based antitumor therapies. In clinical treatments of bladder cancer, BCG therapy is considered the most effective immunotherapy to date (45). Moreover, other than its application in nonspecific immunotherapy for cancer, BCG has also been used as an immune adjuvant for active specific immunotherapy in tumor vaccines in melanoma patients with measurable clinical responses (46, 47). The BCG therapy has often been associated with the activation of NK cells (45, 48). Because both mouse and human DCs activated with BCG are very efficient in activating NK cells (44), the ability of TLR-dependent microbial stimuli to render DCs efficient stimulators of NK cells, even in the presence of exogenous IL-2.

Other cytokines, such as IL-12 and IL-15, have been shown to be involved in NK cell activation (35, 36). In our system we can exclude a role for IL-15, because IL-15-deficient DCs activated with LPS and CpG are able to induce efficient IFN-γ production by NK cells in an IL-2-dependent manner (data not shown). Moreover, concerning IL-12, mouse and human DCs efficiently secrete bioactive IL-12 only if exposed to IL-4 (37). As mentioned in the introduction, two pathways for DC-mediated NK cell activation have been described: one dependent on IL-4, and the other dependent on microbial stimuli (7, 9). IL-4 is, indeed, a DC semimature stimulus, and DCs exposed to IL-4 acquire the ability to activate NK cells independently from the presence of full-activation microbial stimuli and IL-2, although the presence of microbial stimuli increases the efficiency of this process (4). In addition, the exposure of DCs to IL-4 inhibits microbial-induced IL-2 production (38). Thus, it is possible that IL-12 contributes to DC-medi-
References


