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Dendritic Cells and NK Cells Stimulate Bystander T Cell Activation in Response to TLR Agonists through Secretion of IFN-αβ and IFN-γ

Arun T. Kamath, Christopher E. Sheasby, and David F. Tough

Recognition of conserved features of infectious agents by innate pathogen receptors plays an important role in initiating the adaptive immune response. We have investigated early changes occurring among T cells after injection of TLR agonists into mice. Widespread, transient phenotypic activation of both naïve and memory T cells was observed rapidly after injection of molecules acting through TLR3, -4, -7, and -9, but not TLR2. T cell activation was shown to be mediated by a combination of IFN-αβ, secreted by dendritic cells (DCs), and IFN-γ, secreted by NK cells; notably, IFN-γ-secreting NK cells expressed CD11c and copurified with DCs. Production of IFN-γ by NK cells could be stimulated by DCs from TLR agonist-injected mice, and although soluble factors secreted by LPS-stimulated DCs were sufficient to induce IFN-γ, maximal IFN-γ production required both direct contact of NK cells with DCs and DC-secreted cytokines. In vitro, IFN-αβ, IL-18, and IL-12 all contributed to DC stimulation of NK cell IFN-γ, whereas IFN-αβ was shown to be important for induction of T cell bystander activation and NK cell IFN-γ production in vivo. The results delineate a pathway involving innate immune mediators through which TLR agonists trigger bystander activation of T cells. The Journal of Immunology, 2005, 174: 767–776.
Materials and Methods

**Mice and injections**

C57BL/6 (B6) mice were obtained from Charles River-U.K. or the specific pathogen-free unit at the Institute for Animal Health. IFN-γ-deficient (IFN-γ−/−) mice, 129SvEv(129) mice, 129 background mice that were IFN-α/β receptor deficient (IFN-α/βR−/−) (16) (originally purchased from B&K Universal), and DO11.10 TCR transgenic mice (17) (originally obtained from Dr. F. Powrie, Sir William Dunn School of Pathology, University of Oxford, Oxford, U.K.) were obtained from the specific pathogen-free unit at the Institute for Animal Health. C3H/HeN and C3H/HeJ mice were obtained from Harlan U.K. All injections were given i.v. in PBS. All animal experimentation was conducted with the approval of the Home Office and the ethical review committee of the Institute for Animal Health.

**Reagents**

LPS (from *Escherichia coli* 055:B5) and poly I:C were purchased from Sigma-Aldrich. Peptide scan, zymosan, and R-848 were purchased from InvivoGen (San Diego, CA). The sequences for CpG-containing oligonucleotides were supplied by Dr. H. Hochrein (Technische Universität München, Munich, Germany) and were synthesized by MWG Biotech. Oligo2216 (5′-GGG GGA CGA TCG TCGG GGG GG) and control oligo (5′-GGG GGA CGA TCG TCGG GGG GG) were used (phosphothioated bases in bold, CpG motif underlined) (18). Murine IFN-γ protein and rabbit anti-murine Abs against murine IFN-α and IFN-β were purchased from PBL Biomedical Laboratories. mAbs against IFN-γ (clone C17.8) and IFN-β (clone 2F-D3) were purchased from HyCult Biotechnology and Yamasaki, respectively. IFN-γ-protein and mAbs against IFN-γ (clone 37895.11) and IL-18 (clone 93-10C) were obtained from R&D Systems, and mAbs against IL-12p70/p40 (clone C71.8) and TNF-α (clone MP6-XT3) were purchased from BD Biosciences. IL-18 was purchased from BioSource International. Recombinant murine IFN-α-4 was a gift from A. Le Bon (Edward Jenner Institute for Vaccine Research, Compton, U.K.) (19).

**Isolation of DCs and NK cells**

Spleen fragments were digested for 20 min at room temperature with 0.14 mg/ml DNase I (Sigma-Aldrich) and either 1 mg/ml collagenase III (Lorne Laboratories) or 0.4 mg/ml Liberase CI (Roche), then treated for 5 min with 7.9 mM EDTA to disrupt T cell–DC complexes. Low density cells were enriched by centrifugation on Nycoprep 1.077A (AXIS-SHIELD Laboratories) or 0.4 mg/ml Liberase CI (Roche), then treated for 5 min with 0.14 mg/ml trypsin (Roche) and 2% paraformaldehyde, permeabilized with 0.1% saponin, and then stained with IFN-γ, mAbs against CD4, CD44, CD54 (ICAM), CD69, CD86, CD11c, CD49b (DX5) were purchased from BD Biosciences. PE-conjugated KJ1-26, which reacts specifically with the Fcγ receptor deficient (IFN-γ−/−) mice, 129/SvEv(129) mice, 129 background mice that were IFN-α/β receptor deficient (IFN-α/βR−/−) (16) (originally purchased from B&K Universal), and DO11.10 TCR transgenic mice (17) (originally obtained from Dr. F. Powrie, Sir William Dunn School of Pathology, University of Oxford, Oxford, U.K.) were obtained from the specific pathogen-free unit at the Institute for Animal Health. C3H/HeN and C3H/HeJ mice were obtained from Harlan U.K. All injections were given i.v. in PBS. All animal experimentation was conducted with the approval of the Home Office and the ethical review committee of the Institute for Animal Health.

Splenocytes were labeled with anti-CD11c–FITC, and CD11c+ cells were removed by immunomagnetic bead depletion using Dynabeads (Dynal Biotech). Typically, spleens from three to six mice were pooled to generate CD11c+ cells for one experiment. For depletion of CD11c+ cells, splenocytes were labeled with anti-CD11c–FITC, and CD11c− cells were sorted with a MoFlo flow cytometer (DakoCytomation). For depletion of DX5+ cells, DX5++ cells were enriched by centrifugation on Nycoprep 1.077A (AXIS-SHIELD Laboratories) or 0.4 mg/ml Liberase CI (Roche), then treated for 5 min with 0.14 mg/ml trypsin (Roche) and 2% paraformaldehyde, permeabilized with 0.1% saponin, and then stained with IFN-γ, mAbs against CD4, CD44, CD54 (ICAM), CD69, CD86, CD11c, CD49b (DX5) were purchased from BD Biosciences. PE-conjugated KJ1-26, which reacts specifically with the Fcγ receptor deficient (IFN-γ−/−) mice, 129/SvEv(129) mice, 129 background mice that were IFN-α/β receptor deficient (IFN-α/βR−/−) (16) (originally purchased from B&K Universal), and DO11.10 TCR transgenic mice (17) (originally obtained from Dr. F. Powrie, Sir William Dunn School of Pathology, University of Oxford, Oxford, U.K.) were obtained from the specific pathogen-free unit at the Institute for Animal Health. C3H/HeN and C3H/HeJ mice were obtained from Harlan U.K. All injections were given i.v. in PBS. All animal experimentation was conducted with the approval of the Home Office and the ethical review committee of the Institute for Animal Health.

**Cytokine detection**

IFN-γ concentrations in supernatants were measured using mouse IFN-γ DuoSet or Quantikine M ELISA kits (R&D Systems). To detect intracellular IFN-γ, GolgiStop (BD Biosciences) was added to wells for the last 3–4 h of culture. After staining for cell surface markers, cells were fixed in 2% paraformaldehyde, permeabilized with 0.1% saponin, and then stained with anti-IFN-γ–allophycocyanin (BD Biosciences). To measure total IFN activity, an IFN bioassy was used, in which samples were assayed for their ability to protect L929 cells against encephalomyelocardiatis virus infection (21).

**Results**

**LPS induces phenotypic activation of naive and memory phenotype T cells**

Previous work has shown that injection of LPS into mice stimulates selective proliferation of memory phenotype (CD44highCD62Llow) CD8+ T cells, but expression of CD69 on a high proportion of all T cells (11). The latter observation suggests that many T cells receive a partial activation signal in response to LPS. To investigate whether T cells also show other signs of activation in LPS-treated mice, LPS was injected i.v. into normal B6 mice, and the expression of a number of cell surface molecules associated with T cell activation was assessed (Fig. 1A). In addition to CD69, CD54, CD86, CD95, and Ly6A/E were consistently up-regulated on CD4+ and CD8+ T cells present in both LNs and spleen. Phenotypic activation was apparent among CD44high and CD44low cells, indicating that both naive and memory T cells received activation signals (Fig. 1B). Moreover, LPS induced phenotypic activation of TCR transgenic CD4+ T cells when injected into unprimed DO11.10 mice (note that CD4+ T cells in BALB/c background mice are essentially Ly6A/E− in a resting state; Fig. 1C). Because transgenic T cells in these mice (specific for OVA peptide 323–339 in association with I-Ak) (17) are uniformly naive in the absence of immunization with OVA (data not shown), this result provides clear evidence that truly naive T cells receive activation signals in response to LPS in vivo.

The increase in expression of activation molecules after injection of LPS was rapid, being detectable by 4 h, and was maximal by 24 h (Fig. 1D). However, these changes were also transient, as expression of all markers had returned to baseline by 7 days. A similar phenotypic response was observed after injection of LPS isolated from many different bacterial strains (including *E. coli*, *Salmonella enteritidis*, *Pseudomonas aeruginosa*, and *Neisseria meningitidis*) and in mice of several different backgrounds (B6, BALB/c, 129/SvEv, and C3H/HeN; data not shown). T cell expression of activation markers occurred in response to low doses of LPS in vivo, with up-regulation apparent after injection of 10 ng of LPS, and increased in a dose-dependent manner (Fig. 1E). Overall, these experiments indicated that LPS induced widespread phenotypic activation of T cells in vivo.
FIGURE 1. LPS induces phenotypic activation of naive and memory phenotype T cells in vivo. A, B6 mice were injected i.v. with LPS (50 μg) or PBS and were killed 24 h later. Histograms show the expression of the indicated markers on gated CD8^+ or CD4^+ T cells from pooled LN of LPS-injected (thick line) or PBS-injected (thin line) mice. B, B6 mice were injected i.v. with LPS (10 μg) or PBS and were killed 24 h later. Dot plots show CD44 and Ly6A/E staining of gated CD8^+ or CD4^+ T cells from pooled LN cells of PBS-injected (left) or LPS-injected (right) mice. C, D, DO11.10 mice were injected i.v. with LPS (10 μg) or PBS and were killed 24 h later. The histogram shows Ly6A/E expression on gated CD4^+ KJ1–26^+ T cells from PBS-injected (●) or LPS-injected (□) mice. D, B6 mice were injected i.v. with LPS (50 μg) or PBS and were killed 4 h, 24 h, or 7 d later. Data show the average Ly6A/E expression (as mean fluorescence intensity (MFI) ± SE) for three mice per group. E, B6 mice were injected i.v. with PBS or the indicated dose of LPS and were killed 24 h later. Data show the average Ly6A/E expression (as MFI ± SE) for three mice per group.

Cytokines secreted by CD11c^+ cells mediate LPS-induced T cell phenotypic activation

To investigate how LPS stimulates phenotypic activation of T cells in vivo, we first determined whether its effects could be replicated in vitro. In these and subsequent experiments, Ly6A/E was used as a marker of T cell activation, but similar changes were observed for the other markers described in Fig. 1 (data not shown). As shown in Fig. 2A, phenotypic activation of T cells did occur when total splenocytes were cultured overnight in the presence of LPS; concentrations of LPS as low as 0.1 ng/ml were sufficient to induce this effect (data not shown). However, purified T cells cultured in the presence of LPS did not up-regulate activation markers, indicating that LPS acted indirectly to stimulate T cells (Fig. 2A). Moreover, the indirect effects of LPS on T cells were cytokine mediated, because the supernatant of spleen cells obtained from LPS-injected mice (after overnight culture) induced phenotypic activation when added to CD8^+ T cells purified from untreated mice (Fig. 2B).

In considering which cells within the spleen were responsible for production of the T cell stimulatory cytokines, APCs (DCs and macrophages) seemed likely candidates given their key role in the innate recognition of infectious agents and, in particular, their ability to respond to LPS. We used a DC marker, CD11c, to investigate the potential role of these cells in the stimulation of T cells by LPS. Spleen cells were obtained from mice 1 h after LPS injection and depleted of CD11c^+ cells by cell sorting. The remaining CD11c^− cells were then cultured overnight, and the supernatant from these cells was assayed for its ability to induce T cell phenotypic activation. As shown in Fig. 2C, supernatant from CD11c^− cells did not stimulate up-regulation of Ly6A/E when added to purified CD8^+ T cells. In contrast, CD11c^+ cells isolated from the spleens of LPS-injected mice secreted cytokines that induced T cell phenotypic activation (Fig. 2C). Therefore, CD11c^+ cells were necessary and sufficient for production of the T cell stimulatory cytokines in response to LPS. This appeared to be due to direct effects of LPS, because CD11c^+ cells purified from control mice secreted T cell stimulatory cytokines when treated with LPS in vitro (Fig. 2D).

Agonists of several different TLRs trigger CD11c^+ cells to secrete T cell stimulatory cytokines

Previous work has shown that CpG ODNs also induce phenotypic activation of T cells in vivo (14). We investigated whether CpG ODNs as well as agonists of other TLRs acted similarly to LPS in stimulating the production of T cell stimulatory cytokines by CD11c^+ cells. Agonists of TLR2 (zymosan and peptidoglycan) (22, 23), TLR3 (poly I:C) (5), TLR4 (LPS) (3, 4), TLR7 (R848) (24), and TLR9 (CpG ODNs) (15) were injected into mice, and CD11c^+ splenocytes were isolated 1 h later. After culturing these cells overnight, the supernatants were tested for their ability to induce up-regulation of Ly6A/E when added to purified CD8^+ T cells. As shown in Fig. 3A, only zymosan and peptidoglycan failed to induce production of T cell stimulatory cytokines by CD11c^+ cells; an identical pattern of response was observed when in vivo T cell activation was assessed 24 h after injection of the various TLR agonists (Figs. 3B and 4). Therefore, triggering of TLR3, -4, -7,
and -9, but not TLR2, leads to the production of cytokines by CD11c+ cells that induce widespread phenotypic activation of T cells.

**CD11c+ cells induce phenotypic activation of T cells through secretion of IFN-α and IFN-γ**

Previous work has shown that IFN-α/β contributes to the bystander T cell phenotypic activation stimulated by CpG ODNs (14). To determine whether IFN-α/β plays a role in T cell activation induced by LPS-stimulated CD11c+ cells, we examined the ability of T cells from IFN-α/β receptor-deficient (IFN-α/βR-) mice to respond to CD11c+ cell supernatant in vitro. As shown in Fig. 5A, up-regulation of Ly6A/E was greatly reduced on IFN-α/βR- compared with wild-type (WT) T cells, indicating that IFN-α/β contributed to the induction of T cell activation. This type I IFN-dependent effect appeared to be due to both IFN-α and IFN-γ, because neutralizing Abs against either reduced the degree of Ly6A/E up-regulation by WT T cells (Fig. 5B).

Because the phenotypic response of IFN-α/βR- T cells was not completely abrogated, we investigated the consequences of blocking the activity of other cytokines. Addition of neutralizing Abs against TNF-α, the IL-2/IL-15R-chain (CD122), IL-18, or IL-12 to the CD11c cell supernatant had no effect on T cell up-regulation of Ly6A/E (data not shown). Conversely, anti-IFN-γ partially inhibited the T cell response (Fig. 5B). Furthermore, when CD11c+ cells were purified from LPS-injected, IFN-γ-deficient (IFN-γ-) mice, the supernatant derived from these cells had a...
are representative of at least two independent experiments. Expression as the mean fluorescence intensity (MFI) and was measured after 24-h culture. All data show Ly6A/E

The indicated concentrations of IFN-γ were added (at 1/2 dilution) to CD8+ cells (Fig. 5B). Notably, no up-regulation of Ly6A/E was observed when a combination of Abs against IFN-γ and -β were added (Fig. 5C). Taken together, these data indicated that IFN-αβ and IFN-γ accounted for all the T cell stimulatory activity present in the CD11c+ cell supernatants. In agreement with this, IFN-α, -β, and -γ were all capable of stimulating up-regulation of Ly6A/E by purified T cells (Fig. 5C).

To determine whether IFN-αβ and IFN-γ also contribute to TLR agonist-induced T cell activation in vivo, T cell phenotype was examined after injection of poly I:C, LPS, R848, or CpG ODNs into IFN-αβR−/− or IFN-γ−/− mice (Fig. 4). In all cases, up-regulation of Ly6a/E was reduced in both types of knockout mice compared with controls. The extent of the reduction in T cell activation varied with the different agonists; for some, a greater effect was observed in IFN-αβR−/− mice. For example, for R848, Ly6a/E up-regulation was reduced by 82–95% (for CD4+ or CD8+ T cells, respectively) in IFN-αβR−/− mice, but only by ~50% in IFN-γ−/− mice, whereas for CpG ODNs, a greater reduction was observed in IFN-γ−/− than in IFN-αβR−/− mice (44–51 vs 22–37%). Nevertheless, the data indicated that both IFN-αβ and IFN-γ participate in bystander T cell activation induced by all stimuli tested.

IFN-αβ and IFN-γ are produced by distinct cell populations

Although we had used CD11c as a marker to enrich for DCs, the identification of IFN-γ as an active factor in the CD11c+ cell supernatant raised the question of whether DCs were actually responsible for secreting these cytokines. To investigate the identity of the IFN-γ-secreting cells more definitively, we combined intracellular cytokine staining with analysis of additional cell surface molecules on the CD11c+ cell population. As shown in Fig. 6A, staining with the NK cell marker DX5 revealed a population of DX5+ CD11c+ cells; the same cells were also positive for another NK cell marker, NK1.1 (in B6 mice), and for CD11b, but were negative for CD3, CD14, CD19, MHC class II, Gr-1, and F4/80 (data not shown). This phenotype is consistent with these cells being

![FIGURE 4. IFN-αβ and IFN-γ contribute to TLR agonist-induced phenotypic activation of T cells in vivo. A. Comparison of response in WT (129) vs IFN-αβR−/− mice. B. Comparison of response in WT (129) vs IFN-γ−/− mice. Data represent the average Ly6a/E expression on CD8+ (left) or CD4+ (right) T cells (as mean fluorescence intensity (MFI) ± SE) for three mice per group.](http://www.jimmunol.org/)

![FIGURE 5. CD11c+-derived IFN-αβ and IFN-γ induce phenotypic activation of T cells. A. WT (B6) or IFN-γ−/− mice were injected i.v. with LPS (10 μg) or PBS, and CD11c+ cells were purified 1 h later. After overnight culture, supernatants of CD11c+ cells were obtained and added (at 1/2 dilution) to CD8+ T cells from WT (129) or IFN-αβR−/− mice. B. B6 mice were injected i.v. with LPS (10 μg) or PBS, and CD11c+ cells were purified 1 h later. After overnight culture, supernatants of CD11c+ cells were obtained. Neutralizing Abs against the indicated cytokines were added to the CD11c+ supernatants before adding the conditioned medium (at 1/2 final dilution) to CD8+ T cells (from B6 mice). IFN-αβγ. All three Abs were added. C. The indicated concentrations of IFN-α, -β, or -γ were added to purified B6 CD8+ T cells, and Ly6a/E expression was measured after 24-h culture. All data show Ly6a/E expression as the mean fluorescence intensity (MFI) and are representative of at least two independent experiments.](http://www.jimmunol.org/)

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NK cells (25). Strikingly, virtually all IFN-γ-producing cells were contained within the DX5⁺ CD11c⁺ cell population (Fig. 6A).

To determine whether the same cells were also producing IFN-α/β, CD11c⁺ DX5⁻ and DX5⁺ cells from LPS-injected mice were cultured for 24 h, and IFN-α/β activity in the supernatants was measured by bioassay. As shown in Fig. 6B, bioactive IFN was produced predominantly by the CD11c⁺DX5⁻ cell population. Importantly, all the bioactive IFN produced by these cells appeared to be IFN-α/β, because IFN-γ was essentially absent in the supernatant of CD11c⁺DX5⁻ cells (Fig. 7A). Therefore, IFN-α/β and IFN-γ were produced by discrete cell populations after LPS injection: IFN-α/β by DCs and IFN-γ by NK cells.

**TLR agonist-stimulated DCs induce IFN-γ production by NK cells**

The production of IFN-α/β by DCs and IFN-γ by NK cells after LPS injection raised the question of whether these responses were triggered independently or were in some way linked. Consistent with the latter idea, little or no IFN-γ was produced when CD11c⁺ cells from mice injected 1 h previously with LPS were separated into DX5⁺ and DX5⁻ subsets before culture (Fig. 7A). This observation suggested that signals derived from LPS-stimulated DCs were required for triggering NK cell IFN-γ production.

To investigate whether TLR agonist-stimulated DCs were capable of directly inducing NK cells to produce IFN-γ, DCs were purified from LPS-injected mice and mixed with DX5⁺ NK cells from untreated mice; NK cells were labeled with CFSE to permit discrimination between the two cell populations. To eliminate any effects due to possible carryover of LPS from the injected mice, either polymyxin B was added to cultures or NK cells were derived from LPS-hyporesponsive C3H/HeJ mice (26) (in which case these cells were mixed with DCs from LPS-responsive C3H/HeN mice). As shown in Fig. 7B, NK cells mixed with DCs from LPS-injected, but not PBS-injected, mice produced IFN-γ. Similarly, DCs from mice injected with poly I:C, R848, or CpG ODNs stimulated NK IFN-γ expression (Fig. 7C). (Note that no CD11c⁺ cell IFN-γ secretion was detectable after injection of peptidoglycan or zymosan (data not shown).) Direct contact between DCs and NK cells was not essential, because NK cells expressed IFN-γ when separated from LPS-exposed DCs by a porous membrane, although minimal cell contact-independent IFN-γ production was observed in response to other TLR stimuli (Fig. 7, B and C). Interestingly, greater induction of LPS-induced, contact-independent IFN-γ production was observed when cells were derived from C3H as opposed to B6 mice (compare Fig. 7, B and C), indicating that strain differences may exist in this respect. Overall, however,
it was evident that IFN-γ production was much higher when DCs and NK cells were mixed together. Hence, TLR agonist-stimulated DCs provide both soluble and cell-associated factors that play a role in inducing resting NK cells to produce IFN-γ.

**Role for IFN-αβ in DC-induced IFN-γ production**

Because DCs produce type I IFN in response to LPS injection, we investigated the possibility that IFN-αβ contributed to the stimulation of NK cell IFN-γ production. NK cells were obtained from untreated WT (129) or IFN-αβR−/− mice and cultured with DCs from LPS-injected WT mice. In cultures where DCs and NK cells were separated by porous membranes, IFN-αβR−/− NK cells failed to produce IFN-γ, indicating an absolute requirement for IFN-αβ in DC cytokine-induced IFN-γ expression (Fig. 8A). IFN-γ production by IFN-αβR−/− NK cells was also greatly reduced compared with that by WT NK cells when these cells were allowed to come into direct contact with DCs (Fig. 8A), although the response was not completely eliminated. Similarly, addition of neutralizing anti-IFN-αβ to cocultures of WT DCs and WT NK cells partially inhibited IFN-γ expression (Fig. 8B).

The failure to totally abolish NK cell IFN-γ production in DC-NK cell cocultures by blocking IFN-αβR-mediated signaling in vitro implied that IFN-αβ-independent mechanisms also contributed to the response. However, although the approaches described above should block any IFN-αβ stimulation of NK cells, they would not preclude IFN-αβ-mediated effects on DCs themselves, which could occur before placing these cells in culture. To address this possibility, we measured IFN-γ expression when various combinations of IFN-αβR-positive and -negative DCs and NK cells were cocultured (Fig. 8A). A low percentage of NK cells expressed IFN-γ even when both LPS-stimulated DCs and NK cells were derived from IFN-αβR−/− mice, providing definitive evidence that DCs were capable of stimulating IFN-γ production by IFN-αβ-independent mechanisms under these culture conditions. However, it was also evident that the percentage of IFN-γ-expressing NK cells was appreciably reduced when IFN-αβR−/− DCs were used. Therefore, IFN-αβ stimulation of both DCs and NK cells contributes to the induction of NK cell IFN-γ expression.

To determine whether other cytokines acted together with IFN-αβ in DC-stimulated production of NK cell IFN-γ, various neutralizing Abs were added to DC-NK cell cocultures (Fig. 8B). Abs against the IL-2/15R β-chain (CD122) had no effect when added alone or in combination with other Abs, indicating no role for IL-2 or IL-15. Conversely, although anti-IL-12 had no effect on IFN-γ production when added on its own, diminished IFN-γ production was observed when anti-IL-12 was added in combination with anti-IFN-αβ; this result suggested that a positive effect of IL-12 on IFN-γ production was only evident when IFN-αβ activity was blocked, consistent with an antagonistic relationship between these cytokines, as previously reported (27).

Addition of anti-IL-18 alone substantially inhibited NK cell production of IFN-γ, causing a reduction similar to that mediated by anti-IFN-αβ. IFN-γ production was further reduced when a combination of Abs against IFN-αβ and IL-18 was added to cultures, showing that these two cytokines could act independently of each other. Lastly, anti-IL-12 caused an additional decrease in IFN-γ expression when combined with anti-IFN-αβ and anti-IL-18, but not with anti-IL-18 alone, again compatible with the view that IL-12 contributes to LPS-stimulated DC induction of IFN-γ expression only when IFN-αβ activity is blocked. Nearly complete inhibition of IFN-γ production was observed when Abs against IFN-αβ, IL-18, and IL-12 were added. Taken together, the results show a strong requirement for cytokines even in the cell contact-dependent induction of NK cell IFN-γ expression by LPS-stimulated DCs, with IFN-αβ and IL-18 playing predominant roles.

Finally, for each of the TLR agonists that were shown to induce bystander T cell activation, we investigated the importance of IFN-αβ in the in vivo stimulation of IFN-γ production. Poly I:C, LPS, R848, or CpG ODNs was injected into IFN-αβR−/− or control mice, and 1 h later splenic CD11c+ cells were purified and placed in culture. Supernatants obtained after overnight culture were then assayed for IFN-γ by ELISA (Fig. 9). Equivalent

![Figure 8](http://www.jimmunol.org/Downloadedfrom/773TheJournalofImmunology)

**FIGURE 8.** IFN-αβ and IL-18 contribute to DC-stimulated NK cell IFN-γ production. LPS (10 μg) was injected i.v. into WT (129) or IFN-αβR−/− mice (A) or B6 mice (B), and CD11c+ cells were purified 20 min later. CD11c+ cells were placed in culture with CFSE+ DX5+ cells from untreated WT or IFN-αβR−/− mice (A) or B6 mice (B). A, CD11c+ and DX5+ cells were either mixed together in the same well or separated by a porous membrane in Transwells as indicated. B, Neutralizing Abs against the indicated cytokines were added to cocultures of CD11c+ and CFSE+ DX5+ cells. Data show the percentage of IFN-γ+ cells among DX5+ CFSE+ cells (mean ± SE from two (A) or three (B) experiments).
amounts of IFN-γ were produced after injection of CpG ODNs into either WT or IFN-αR−/− mice, indicating that this TLR9 agonist was capable of stimulating IFN-γ expression by an IFN-αβ-independent mechanism. Conversely, IFN-γ induction by poly I:C, LPS, and R848 was markedly reduced in IFN-αR−/− mice. Poly I:C stimulated only low level IFN-γ production (~100 pg/ml) in WT mice on a 129 background and completely failed to induce IFN-γ in IFN-αR−/− mice. LPS and R848 each stimulated much higher amounts of IFN-γ in WT mice (1–2 ng/ml), but this was reduced by >90% in IFN-αR−/− mice. Therefore, in vivo stimulation of NK cell IFN-γ expression by agonists of TLR3, -4, and -7, but not TLR9, is highly dependent on IFN-αβ.

**Discussion**

T cell responses are regulated indirectly by the triggering of pathogen recognition receptors, mainly through the downstream effects of such signals on APCs. TLR-induced maturation of DCs alters their ability to stimulate and modulate the function of T cells responding to specific Ag. In this paper we demonstrate that TLR-mediated stimulation of DCs can also lead to global, non-Ag-specific changes in T cells, namely, the widespread expression of NKG2D ligands by converting NKG2D to a more active form (52), which might enhance the ability of NK cells to respond to DNA viruses such as MCMV are mediated in part through TLR9 recognition of viral DNA (32, 33).

Recently, a number of papers have reported that DCs can stimulate NK cell activation in vitro (34–49). In such studies the dependency of NK cell activation on direct contact between DCs and NK cells has varied. A strict requirement for cell-cell contact was demonstrated in one study of mouse NK cells (34), whereas activation of human NK cells by DCs has been shown to be dependent on (36, 38–40, 42) or independent of (35, 44) direct cell contact in different studies. Many factors might influence these results, including the source of DCs and the timing and nature of the specific maturation stimulus. For example, in a previous analysis of mouse NK cells where a strict requirement for cell-cell contact in NK activation was observed (34), a DC cell line (D1), stimulated by culture with TNF-α for 24 h, was used. As shown in this study, the extent to which splenic DC activation of NK cells is dependent on direct cell-cell contact differs when stimuli acting through different TLRs are used and may also depend on the genetic background of the host. In any case, the present results demonstrate that LPS-stimulated mouse DCs can trigger activation of resting NK cells in a cell contact-independent manner, which is highly dependent on IFN-αβ.

Direct contact with LPS-stimulated DCs induced greater IFN-γ production by NK cells than soluble factors alone. Importantly, however, this cell contact-mediated NK stimulation was also dependent on cytokines, with IFN-αβ, IL-18, and IL-12 all contributing; blocking the combined activity of these cytokines almost completely abrogated IFN-γ expression. Interestingly, a role for IL-12 in the stimulation of NK cells by LPS-activated DCs was only observed when IFN-αβ activity was neutralized. This might be due to an inhibition of IL-12 production by IFN-αβ, a phenomenon that has been previously observed in the context of viral infection (33, 50, 51).

IFN-αβ, IL-12, and IL-18 were also involved in NK cell stimulation by DCs triggered with agonists of TLR3, -7, or -9, although (as alluded to above) the relative role of each cytokine varied with the specific stimulus (data not shown). Conversely, Abs against CD122 had no effect on IFN-γ production (either when added alone or in combination with other Abs; data not shown), implying that there was no role for IL-2 or IL-15. This finding contrasts with the results of recent papers, in which IL-15 (49) or IL-2 (48) was reported to be required for NK cell activation by mouse DCs (49). These differences might be accounted for by the use of in vitro generated, bone marrow-derived DCs, rather than splenic DCs in these other studies and/or the use of different DC activation stimuli (in one case, in vitro exposure of DCs to LPS (49); in the other, treatment with E. coli (48)).

For IFN-αβ at least, enhancement of NK cell activation was due to effects on both NK cells and DCs, because reduced IFN-γ production was observed when either cell type was derived from IFN-αβR−/− mice. One way in which IFN-αβ might promote the cell contact-dependent activation of NK cells by DCs would be through induction of stimulatory cell surface molecules on DCs. Evidence supporting this idea has come from a study of human DCs (44). In that paper it was shown that IFN-α induces DC expression of MHC class I chain-related proteins A and B (MICA and MICB), which are ligands for the NK cell-activating receptor NKG2D, and that these molecules were required for cell contact-dependent stimulation of NK cells by IFN-α-stimulated human DCs. At present, though, it is unknown whether IFN-αβ induces expression of ligands for NK cell-activating receptors on mouse DCs. Interestingly, there is also indirect evidence suggesting that IFN-αβ might enhance the ability of NK cells to respond to NKG2D ligands by converting NKG2D to a more active form (52),
suggesting one mechanism by which IFN-αβ could act on NK cells to enhance cell contact-dependent stimulation. In addition, because IFN-αβ has been shown to stimulate human DCs to produce NK-attracting chemokines in response to microbial stimuli (53), a deficit in LPS-induced recruitment of NK cells by DCs may contribute to the reduction in IFN-γ induction observed in IFN-αβ−/− mice.

The key contribution of IFN-αβ to TLR agonist-induced NK cell and T cell activation raises the question of whether this response is mediated by a specific DC subpopulation. In view of data showing that some TLRs are differentially expressed among DC subsets (54), the answer may well depend on which agonist is used. Dalod et al. (43) found that the plasmacytoid DC (PDC) population was chiefly responsible for NK cell activation after MCMV infection. In the experiments described in this study, total CD11c+ cells include all major splenic DC populations, including PDCs. However, our preliminary results suggest that PDCs are not required for LPS-stimulated bystander activation (A. T. Kamath and D. F. Tough, unpublished observations). Additional investigation will be necessary to delineate the specific relationship between agonists of particular TLRs and individual DC subpopulations in bystander activation.

IFN-αβ and IFN-γ have also been shown to stimulate bystander proliferation of memory phenotype CD8+ T cells in vivo (10, 55). However, the proliferative bystander response differs from the phenotypic activation described in this study in two respects. First, phenotypic activation applies to the majority of both CD4+ and CD8+ T cells, including naive cells, whereas proliferation is restricted to CD44high CD8+ T cells. Second, whereas IFN-αβ and IFN-γ act directly on T cells to induce phenotypic activation, stimulation of purified T cells with IFN does not initiate CD44high CD8+ T cell division. Rather, proliferation appears to be driven by IL-15, which can be induced by IFN-αβ and IFN-γ (12, 13). How the indirect, IL-15-driven proliferation of CD44high CD8+ T cells is influenced in vivo by the direct, partially activating effects of IFN-αβ and IFN-γ is unknown.

T cell phenotypic activation in vivo was shown to occur in response to the triggering of several different TLRs, implying that non-Ag-specific activation of T cells is likely to be a common response to infection. Molecules acting through TLR3, -4, -7, and -9, but not TLR2 induced this response. Like LPS, poly I:C, R848, and CpG ODNs also triggered DCs to stimulate NK cell activation, whereas no IFN-γ production was observed after treatment with peptidoglycan or zymosan (data not shown). Notably, LPS, poly I:C, R848, and CpG ODNs are all able to induce the expression of IFN-αβ, whereas TLR2 agonists fail to elicit this response (24, 56–60). Future studies will be required to determine whether differences in the induction of bystander activation impact on the nature of the immune response elicited by different adjuvant compounds.

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References