Primed Antigen-Specific CD4+ T Cells Are Required for NK Cell Activation In Vivo upon *Leishmania major* Infection

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Natural killer cells are important innate immune effector cells that actively participate in the early host response to infections, notably through the release of immunoregulatory cytokines and chemokines (1–4). NK cells rapidly produce IFN-γ, which is crucial for pathogen clearance (3, 5) and also critical for the development of subsequent adaptive responses (3). In *Leishmania major* infection in mice, this cytokine was shown to contribute to the development of protective Th1 responses (6–8). It was speculated that NK cells located in draining lymph nodes (dLNs) would exert this role because they could be in close proximity to activated dendritic cells (DCs) and T cells (9–11). Consistent with this, we previously showed that upon *L. major* infection, NK cells were rapidly recruited from the blood to the dLNs, where they secreted IFN-γ in the vicinity of DCs and T cells getting primed (12). NK cells therefore localize in the strategic area, where the initiation of adaptive immune responses occurs, supporting the view that they can influence the polarization of T cell responses (8, 9, 11, 12).

The mechanisms regulating the initiation of NK cell responses in vivo are still incompletely understood. Recent data revealed that, to acquire effector functions, naive NK cells must be primed through a process that requires trans presentation of IL-15 by DCs in LNs (13, 14) or IL-18 (15). Primed NK cells are not fully activated but can kill or secrete cytokines subsequent to additional stimulation provided by pathogens, cytokines, and/or accessory cells. Some pathogens directly activate NK cells upon NK receptor ligation or TLR stimulation (16–19). But it is believed that DCs are the most potent stimulators of NK cells (10, 20, 21) and are required for in vivo NK cell activation (21). In *L. major* infection, NK cell activation was shown to depend on the secretion of IL-12 by myeloid DCs through a TLR9-dependent pathway but not on the release of type I IFNs by plasmacytoid DCs (22–24). Besides DCs, other accessory cells such as monocytes and macrophages can activate NK cells (25). Such a role has not been clearly demonstrated for T cells in vivo.

IL-2 is a cytokine that stimulates NK cells in vitro. In vivo, IL-2 is mainly produced by activated T lymphocytes and is secreted after TCR and costimulatory molecules engagement (26). The contribution of T cell-derived IL-2 to IFN-γ production by NK cells has been poorly established (11, 27). It is believed that NK cells get activated before T cells, and this would preclude such a role. However, studies on T cell activation in dLNs of immunized mice have shown that IL-2 mRNA and proteins can be detected in naive T cells as early as 6 h postimmunization (28, 29). In *L. major* infection, a peak of IFN-γ secretion by NK cells was measured 12 h postinoculation (p.i.), and IFN-γ–secreting NK cells were visualized in the paracortex of dLNs in close proximity to Ag-specific T cells (12). We therefore hypothesized that newly primed Ag-specific CD4+ T cells could contribute to the activation of NK cells to secrete IFN-γ, and we set up experiments to demonstrate such a role.

**Materials and Methods**

**Mice and parasites**

BALB/cByJ and C57BL/6J mice were purchased from Centre d’Elevage Janvier (Le Genest-Saint-Ise, France). C57BL/6 I-A<sup>b</sup>I-B<sup>b</sup> mice were obtained from the Centre de Distribution, Typage et Archivage animal (Orléans, France). BALB/c RAG-1<sup>−/−</sup> and *CITA*<sup>−/−</sup> mice were kindly provided by G. Lauvau (Institut National de la Santé et de la Recherche Médicale, Sophia-Antipolis, France) and BALB/c SCID IL-2<sup>−/−</sup> mice by B. Salomon (Hôpital de la Pitié-Salpêtrière, Paris, France). BALB/c WT 15 and DO11.10 TCR transgenic mice have been previously described (30, 31). They were crossed with RAG-1<sup>−/−</sup> mice, and offspring were further intercrossed to select and derive WT15 and DO11.10 RAG-1<sup>−/−</sup> strains, respectively, referred to as WT15 and DO11.10 in the manuscript. The

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same strategy was used with WT15 RAG-1−/− mice and SCID IL-2−/− mice to generate WT15 RAG-1−/− IL-2−/− animals, referred as WT15 IL-2−/− in the text. The exclusion of mutant SCID alleles was reached through standard PCR and restriction enzymes digestion procedures (http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objecttype=protocol&protocol_id=149). Animals were bred and housed under specific pathogen-free conditions. L. major promastigotes (WHOM/IR/-/173) were grown in M199 medium supplemented with HEPES 25 mM and 20% FCS. Mice were infected with 2 × 10^6 stationary phase promastigotes in the ear dermis. All experiments were performed in compliance with institutional guidelines and have been approved by the regional committee for animal experimentation (CREEA, Nice Côte d’Azur, France).

**Cell suspensions and flow cytometry staining**

LNs were collected and incubated at 37°C for 20 min in RPMI 1640 medium (BioWhittaker, Emerainville, France) containing 1% FCS and 4000 U/ml collagenase I (Invitrogen, Cergy Pontoise, France). Isolated cells were directly stained without any restimulation step. The following mAbs were purchased from BD Biosciences (Le Pont de Claix, France): anti-CD3 (145-2C11), anti-CD4 (RM4.5), anti-CD49b (DX5), anti-CD11c (HL3), anti-CD69 (H1.2F3), anti–IFN-γ (XMG1.2), anti–IL-2 (JES6-5H4), and anti–IL-12p40 (C15.6). For intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s instruction. Cells were analyzed by an FACSCalibur and data processed using CellQuest Pro software (BD Biosciences). The fluorescence threshold was set at a level of 1% in the contralateral PBS-treated LN cells.

**In vivo depletion and neutralization experiments**

For CD4 and CD8 depletion, mice were treated i.p. with three daily injections of 100 μg anti-CD4 (GK1.5) or anti-CD8β (H35) mAb. In the neutralization experiments, single injections of 0.5 mg anti-I-Aβ/Eβ (M5/114) mAb, anti–IL-2 (JES6-5H4), anti–IL-12 (C17.8) or anti-CD40L (MR1) mAbs were produced in-house, whereas anti–IL-12 (C17.8) was prepared in-house. In neutralizing experiments, IFN-γ levels were measured in supernatants by ELISA (eBioscience, Montrouge, France). For kinetic studies, simultaneous detection of IL-2, IL-12, and IFN-γ cytokines in supernatants was obtained using CBA Flex Sets (BD Biosciences). Peripheral LN cells from WT15 mice were also stimulated with rIL-2 and IL-12 (R&D Systems). GolgiStop (BD Biosciences) was added 4 h preanalyses.

**In vitro MHC class II dimer stimulation assays**

Peripheral LN cells from WT15 RAG-1−/− mice were harvested and labeled with anti-CD3 and anti-CD49b mAbs. NK cells (CD3−CD49b+) and T cells (CD3+CD49b−) were sorted by flow cytometry on a FACSVantage SE cell sorter (BD Biosciences). The purity was >95%. Purified NK cells (5 × 10^4/well) and/or T cells (5 × 10^4) were distributed in 96 flat-bottom wells coated with I-Aβ/LACK or I-Aβ/gp63 soluble MHC class II dimers (20 ng/well) and anti-CD28 mAb (500 ng/well). GolgiStop (BD Biosciences) was added 4 h after cytokine stimulation.

**In vitro transfer of CD4+ T cells**

Peripheral LNs and splenic CD4+ T cells were negatively selected using a Mouse CD4 Negative Isolation Kit (Dynal, Invitrogen). Purity was >85%. Unless specified, 10^6 CD4+ T cells were injected i.v. into recipient mice 24 h before L. major infection.

**In vitro peptide stimulation assays**

Peripheral LN cells from WT15 RAG-1−/− mice were plated at 10^6/ml in a 96-well flat-bottom tissue-culture plate in complete media consisting of RPMI 1640 with 2 mM L-glutamine (BioWhittaker) supplemented with 10% FCS (Perbio, Thermo Fisher Scientific, Brebiere, France), penicillin-streptomycin (100 IU/ml penicillin and 100 μg/ml streptomycin; BioWhittaker), and 50 μg 2-ME (BioWhittaker) at 37°C with 5% CO2. The cells were stimulated with 1 μM LACK peptide (aa 156–173: ICFSPSLEHPYVSGSWD) or OVA peptide (aa 323–339: ISQAVHAAHAEINEAGR) (Mimotopes, Clayton Victoria, Australia). Endotoxin levels were <125 pg/ml using Limulus Amebocyte Lysate assay (Cambrex, Paris, France). The following neutralizing and isotype control mAbs (10 μg/ml) were added before peptide stimulation: rat IgG2a from R&D Systems (Lille, France) anti–IL-2 (S4B6); and anti–IL-12 (C17.8) prepared in-house. In neutralizing experiments, IFN-γ levels were measured in supernatants by ELISA (eBioscience, Mortrouge, France). For kinetic studies, simultaneous detection of IL-2, IL-12, and IFN-γ cytokines in supernatants was obtained using CBA Flex Sets (BD Biosciences). Peripheral LN cells from WT15 mice were also stimulated with rIL-2 and IL-12 (R&D Systems). GolgiStop (BD Biosciences) was added 4 h preanalyses.

**FIGURE 1.** CD4+ T cells are required for early production of IFN-γ by NK cells. WT, CD4-deficient mice (A, B) and PBS, anti-CD8β, or anti-CD4-treated WT mice (C, D) were infected with L. major, and dLN cells were harvested and labeled. A and C. Representative intracellular flow cytometry staining for IFN-γ secretion by gated CD3−CD49b− NK cells. The frequency of IFN-γ-expressing cells among total NK cells is indicated. The fluorescence threshold was set at a level of 1% in the contralateral PBS-treated LN cells. B and D, Frequency of IFN-γ-expressing cells among gated NK cells. The number of mice per group is indicated. **p < 0.01; ***p < 0.001 (Mann-Whitney U test).
before intracellular flow cytometry staining 12 h poststimulation. I-A^d/LACK and I-A^b/gp63 were produced as previously described (32).

Statistical analyses

Statistical analyses were performed using nonparametric Mann-Whitney U tests and StatEL software (Ad Science, Paris, France).

Results

Early production of IFN-γ by NK cells is abrogated in mice deficient in CD4^+ T cells

To address the role of CD4^+ T cells in the early activation of NK cells in LNs, we first monitored NK cell activation in several mouse strains deficient in CD4^+ T cells. L. major was inoculated in the ear dermis. dLNs were harvested 12 h later and were directly stained for intracellular IFN-γ. As previously reported (12), NK cells were recruited to the dLNs of BALB/c and C57BL/6 mice, where they secreted IFN-γ 12 h p.i. (Fig. 1, A, B). By contrast, the frequency and number of IFN-γ–secreting NK cells were drastically reduced in dLNs of CD4^+ T cell-deficient RAG-1^−/−, CIITA^−/−, and I-A^b/−/− mice (Fig. 1, A, B, Supplemental Fig. 1A). We excluded a role for NK cells and B cells as infection of CD1d^−/− and μMT mice did not significantly affect IFN-γ secretion by NK cells (Supplemental Fig. 1B, 1C). In addition, the frequency and number of IFN-γ–secreting NK cells were severely reduced upon CD4 but not upon CD8 depletion (Fig. 1C, 1D, Supplemental Fig. 1D). Collectively, our data indicated that CD4^+ T cells were required for early NK cell activation in dLNs following L. major infection.

NK cell activation by Ag-specific CD4^+ T cells

To assess whether NK cell activation in dLNs was controlled by activated parasite-specific CD4^+ T cells, we used WT15 TCR transgenic (Tg) RAG-1^−/− mice that express a TCR specific for the dominant L. major Ag LACK peptide (30) and, as control, DO11.10 TCR Tg Tg mice that bear an OVA-specific TCR (31). Inoculation of parasites in WT15 mice led to a 3–5-fold increase in the frequency and number of IFN-γ–secreting NK cells in dLNs compared with wild-type (WT) mice (Fig. 2A, 2B). The mean fluorescence intensity was also increased, indicating that individual NK cells secreted a higher amount of IFN-γ. By contrast, NK cell activation was minimal in DO11.10 mice bearing an irrelevant T cell compartment and was similar to RAG-1^−/− mice (Fig. 1A, 2B). In addition, a significant increase of NK cells secreting IFN-γ was measured in mice adoptively transferred with purified WT15 but not DO11.10 CD4^+ T cells compared with WT mice (Fig. 2C). Similarly, transfer of WT15 cells into DO11.10 mice induced IFN-γ secretion by NK cells (Supplemental Fig. 2A). Interestingly, as low as 100 to 1000 transferred WT15 cells that homed into the dLNs were able to initiate NK cell activation (Supplemental Fig. 2B). Finally, injection of blocking anti-MHC class II I-A^d/−/− mice in BALB/c mice led to a significant decrease of IFN-γ–secreting NK cells in dLNs (Fig. 2D).

 Altogether, these results demonstrated that newly primed L. major-specific CD4^+ T cells were required for NK cell activation in dLNs early p.i. of the parasite. Activation of Ag-specific CD4^+ T cells therefore precedes and is required to initiate NK cell secretion of IFN-γ in dLNs.

IL-2 and IL-12 regulate NK cell activation

To identify the molecular mechanisms that led to early NK cell activation, we set up an in vitro assay in which naïve LN cells from WT15 mice were stimulated with LACK or irrelevant OVA peptides in the presence or absence of neutralizing Abs. In vitro stimulation with LACK but not OVA peptide led to NK cell secretion of IFN-γ (Fig. 3A). NK cells were the first cells to secrete IFN-γ 12 h poststimulation, and activated CD4^+ T cells became the main producers afterward. Blockade of MHC class II significantly decreased NK cell secretion of IFN-γ, confirming a requirement for Ag presentation and T cell activation (Supplemental Fig. 3A). Neutralization of IL-1, IL-4, IL-10, IL-18, IL-23, IFN-α/β, and IFN-γ signaling pathways or NKG2D and CD94/NKG2 receptors did not affect NK cell secretion of IFN-γ (data not shown). By contrast, the neutralization of IL-2 and/or IL-12 significantly reduced the amount of IFN-γ released, with an
additive effect of the two cytokines confirmed when adding rIL-2 and/or IL-12 (Fig 3B,3C, Supplemental Fig. 3B). Interestingly, NK cells upregulated CD25 upon activation by LACK, allowing them to rapidly respond to low concentrations of IL-2 (Supplemental Fig. 3C). Neutralization of IL-2 receptors had a similar effect as neutralization of the cytokine (Supplemental Fig. 3D). Because DCs were recently shown to prime NK cells by trans-presenting IL-15, we neutralized IL-2 receptors but did not see any decrease of NK cell activation (Supplemental Fig. 3E). Finally, IL-2 was first secreted at 6 h poststimulation by CD4+ T cells, whereas IL-12 was produced by DCs at 12 h (Fig. 3D,3E).

Collectively, our in vitro studies identified a role for both IL-2 secreted by CD4+ T cells and IL-12 produced by DCs in the initiation of NK cell activation.

**IL-2 secreted by Ag-specific CD4+ T cells is required to induce early IFN-γ secretion by NK cells in vivo**

Our studies thus far had revealed that in the absence of Ag-specific CD4+ T cells and despite the presence of DCs, NK cells did not secrete IFN-γ in dLNs of *L. major*-infected mice. T cells may be required to directly activate NK cells and/or to regulate IL-12 secretion by DCs.

We first tested whether CD4+ T cells could directly activate NK cells. We used soluble I-A^d^ dimers covalently linked to LACK or control gp63 peptides to activate T cells (32). FACS-sorted CD4+ T cells and NK cells from uninfected WT15 LNs were cocultured with these peptide–MHC complexes and anti-CD28 mAbs. Although NK cells alone in the presence of either I-A^d^/LACK or I-A^d^/gp63 dimers did not get activated, NK cells incubated with WT15 cells secreted IFN-γ in the presence of I-A^d^/LACK but not of I-A^d^/gp63 dimers. The secretion of IFN-γ by NK cells was associated with T cell activation (Fig. 4A).

We then tested whether IL-2 secreted by newly primed CD4+ T cells was required to activate NK cells in vivo. For this purpose, we transferred purified CD4+ T cells from either IL-2^+/+^ or IL-2^−/−^ WT15 animals. Whereas NK cells from dLNs of infected WT mice transferred with IL-2^+/+^ WT15 cells were able to secrete signi-
IL-2 in vivo, increased the biological activity of endogenous IL-2 through the formation of immune complexes (33). But although anti–IL-2 mAb S4B6 stimulates NK cells, anti–IL-2 mAb JES6-1A12 does not and solely activates regulatory T cells (Tregs) (34). We therefore used JES6-1A12 to neutralize IL-2 in vivo. Interestingly, we found that the neutralization of either IL-2 or IL-12 alone only slightly inhibited NK cell activation (Fig. 6A, Supplemental Fig. 5A). In contrast, the simultaneous neutralization of IL-2 and IL-12 resulted in a significant decrease of the proportion and number of IFN-γ-secreting NK cells in dLN (Fig. 6B). To rule out a role of Tregs that could be activated by JES6-1A12, we injected an anti-CD25 PC61 known to block the function of Tregs (35) either alone or together with the anti–IL-2 mAb. None of these treatments decreased NK cell activation (Supplemental Fig. 5B). Therefore, activated Tregs did not seem to regulate the initiation of NK cell activation.

Then, to link the requirement for Ag-specific CD4+ T cells with the contribution of both IL-2 and IL-12 in early NK cell activation, we hypothesized that T cells might be required to regulate IL-12 secretion by DCs. In support of this view, it has previously been shown that activated CD4+ T cells upregulate CD40L, which interacts with CD40 on DCs, contributing to their maturation and secretion of IL-12. Reciprocally, CD40L engagement on CD4+ T cells potentiates their activation (36, 37). The neutralization of CD40/CD40L interaction led to a significant decrease in the frequency and number of NK cells secreting IFN-γ but also of DCs secreting IL-12 in dLN of L. major-infected mice (Fig. 6B, 6C). These results highlighted the crucial role of CD40/CD40L interaction in linking T cell-derived IL-2 to DCs secreting IL-12 in vivo.

FIGURE 4. Ag-specific CD4+ T cell-derived IL-2 activate NK cells in vitro. A. Purified NK and CD4+ T cells from naive WT15 mice were cultured alone or together in wells coated with I-A^d/LACK or irrelevant I-A^d/gp63 dimers and anti-CD28 mAb. Data show a representative intracellular flow cytometry staining for IFN-γ secretion by gated CD3+ CD4+ T cells, for CD69 cell-surface expression (middle panels), and for IL-2 secretion (right panels) by gated CD3+ T cells. The frequencies of IFN-γ-secreting cells among total NK and of CD4+ T cells are indicated. B. Purified NK and WT15 Tg CD4+ T cells were cultured in wells coated with I-A^d LACK dimers and anti-CD28 mAb in the presence of isotype, anti–IL-12, or anti–IL-2 mAbs. Representative intracellular flow cytometry staining for IFN-γ secretion by gated CD3+ CD49b+ NK cells is shown. The frequency of IFN-γ-expressing cells among total NK cells is indicated. Data are representative of two independent experiments.

FIGURE 5. IL-2 secreted by Ag-specific CD4+ T cells is required to activate NK cells in vivo. Purified CD4+ T cells from IL-2^+/+ and IL-2^−/− WT15 mice were transferred into naive WT mice (A–C) or RAG-1^−/− mice (D). One day later, transferred and nontransferred animals were infected with L. major and dLN and ndLN cells were harvested 12 h p.i. A and D. Frequency of IFN-γ-expressing cells among gated CD3+ CD49b+ NK cells from dLN of recipient mice. B. Representative CD69 cell surface expression on transferred IL-2^+/+ (right panel) or IL-2^−/− (left panel) WT15 Tg T cells from dLN (solid line) and ndLN (dotted line). Data are representative of two independent experiments. C. Number of IL-2-expressing WT15 Tg cells in dLN from recipient mice. A, C, and D. The number of mice per group is indicated. *p < 0.05; **p < 0.01; ***pp < 0.001 (Mann-Whitney U test).
interaction in regulating IL-12 secretion by DCs and demonstrated that the control of the initiation of NK cell activation by Ag-specific CD4+ T cells was not only IL-2 but also CD40/CD40L-dependent. They suggest that T cells regulate DC-mediated NK cell activation in dLNs of *L. major*-infected mice (Fig. 7). As NK cells were also reported to express CD40L, its interaction with CD40 on DCs may also play a role (38, 39).

**Discussion**

The notion currently prevailing is that NK cells are rapidly activated in vivo before cognate T cell activation and that they influence the development of adaptive immune responses. We show in this study that priming of Ag-specific CD4+ T cells is required to initiate NK cell activation, clearly demonstrating that adaptive immune responses also shape innate responses. Our work unraveled a so far unappreciated role for Ag-specific CD4+ T cells in the initiation of the activation of NK cells in vivo.

We also provide the first demonstration of a role for IL-2 secreted by newly primed Ag-responsive CD4+ T cells. IL-2 is a well-

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**FIGURE 6.** The initiation of NK cell activation in vivo is regulated by IL-2, and IL-12 and is dependent on CD40/CD40L interaction. A single dose of neutralizing anti–IL-2 (JES6-1A12) and/or anti–IL-12 (C17.8) or blocking anti-CD40L (MR1) mAbs were injected into WT BALB/c mice 24 h before *L. major* infection. dLN cells were harvested 12 h p.i. A, Frequency of IFN-γ-expressing cells among gated CD3+ CD49b+ NK cells from dLNs of WT mice pretreated with PBS, anti–IL-2, anti–IL-12, or anti–IL-2 plus anti–IL-12 mAbs. B, Frequency and number of IFN-γ-expressing cells among gated NK cells from dLNs of WT mice pretreated with PBS or anti-CD40L. C, Frequency and number of IL-12p40-expressing cells among gated CD3+ CD11chigh DCs from dLNs of WT mice pretreated with PBS or anti-CD40L. The number of mice per group is indicated. *p < 0.05; **p < 0.01; ***p < 0.001 (Mann-Whitney U test).

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**FIGURE 7.** Model for the initiation of NK cell activation in vivo in dLNs of *L. major*-infected mice. A, Following inoculation of *L. major* in the mice ear dermis, naive Ag-specific CD4+ T cells encounter DCs presenting *L. major*-derived peptides in dLNs. At this very early time point, resident NK cells express the intermediate affinity IL-2R and do not secrete IFN-γ. B, Ag-specific CD4+ T cells primed by DCs release IL-2 and upregulate CD40L and CD25, thereby inducing expression of the high-affinity IL-2R. Concomitantly, IL-2 secreted by activated T cells could directly activate NK cells. This activation induces CD25 expression, rendering NK cells more responsive to IL-2. In addition, activated CD4+ T cells could contribute to further maturation of DCs through a CD40/CD40L-dependent mechanism. This leads to the release of IL-12 by DCs, which synergizes with IL-2 to activate NK cells to secrete IFN-γ.
known stimulator of NK cells in vitro (40), but no clear evidence that T cell-derived IL-2 could stimulate NK cells in vivo has so far been provided (11, 27). Using the transfer of IL-2−/− WT15 cells and the in vivo neutralization of IL-2 and IL-12, we found that IL-2 secreted by CD4+ T cells was required to initiate NK cell activation in dLNs 12 h p.i. These data are consistent with kinetics studies that have detected transient IL-2 secretion by Ag-responsive CD4+ T cells 6–8 h post immunization (28, 29, 41, 42) and Ag presentation as early as 2 h to 4 h following i.v. and s.c. injection of Ag (43–46). Thus, priming of Ag-responsive CD4+ T cells can occur very early on, before NK cell activation. How can a low frequency of Ag-activated CD4+ T cells secreting IL-2 control the initiation of NK cell activation? Several mechanisms may explain this observation. CD25 was upregulated on NK cells upon activation, indicating that an amplification loop operated in which NK cells became more responsive to lower concentrations of IL-2 by the acquisition of the high-affinity IL-2R (26). Furthermore, unlike Ag-specific T cells that form long-lasting interactions with DCs during activation in LNs (47), NK cells remain highly motile (48). Such behavior may allow NK cells to continuously sample their microenvironment, resulting in a better integration of signals delivered by IL-2 and IL-12. Lastly, IL-2 may also be required to regulate IL-12 secretion by DCs, thus amplifying DC-mediated NK cell activation. This is consistent with the contribution of IL-2 to the activation of T cells and the upregulation of CD40L expression upon IL-2 treatment (49).

It has been established that DCs play a central role in the activation of NK cells (10, 22–24). In many infections including L. major, IL-12 secreted by DCs has been reported to be the main inducer of IFN-γ secretion by NK cells (50–53). In our study, we found that DCs and IL-12 alone were not sufficient to initiate NK cell activation but that newly primed CD4+ T cells secreting IL-2 were also required. This apparent discrepancy could be explained in a sequential model (Fig. 7). naïve Ag-specific CD4+ T cells primed by DCs presenting MHC class II/peptide complexes release IL-2 and upregulate CD40L expression upon IL-2 treatment (49).

Our results suggest that IL-2 may stimulate rather than prime NK cells. This indicated that neither L. major directly nor TLR triggering on DCs alone were sufficient to induce IFN-γ secretion by NK cells but that it also required CD4+ T cells. Accordingly, we found a similar activation of NK cells in dLNs from L. major-infected MyD88−/− mice compared with WT mice 12 h p.i. (Supplemental Fig. 5C). In other models, DCs have been shown to be involved in the priming of NK cells (13–15). In particular, in a model in which DCs were activated in an Ag-independent way, NK cell activation required IL-15 trans presentation by DCs in LNs (13).

These results suggest that IL-2 may stimulate rather than prime NK cells. But it remains to be determined whether a first step of priming is required. Although we did not find any contribution of IL-15 in vitro, we cannot exclude that resident NK cells in LNs from naive animals are already primed. Technical limitations due to short viability of NK cells in the absence of IL-15 prevented us from testing its role in vivo. By contrast, we could exclude a role of IL-18 as NK cell activation was identical in MyD88−/− mice and WT mice, MyD88 being required for IL-18R signaling (Supplemental Fig. 5C).

We found that a similar mechanism operating during L. major infection and following immunization with OVA. Indeed, NK cells secreted IFN-γ in dLNs of mice transferred with OVA-specific DO11.10 CD4+ T cells and not in WT mice 12 h post immunization with OVA. These T cells were activated and secreted IL-2 in dLNs but not in nondraining LNs (ndLNs) (Supplemental Fig. 6A, 6B). These results suggest that the control of the initiation of NK cell activation in dLNs by newly primed Ag-specific CD4+ T cells may not be restricted to L. major infection. Interestingly, recent studies suggested that CD4+ T cell control of NK cell activation could also be crucial for tumor rejection (54, 55). Furthermore, incubation of human PBMCs with influenza A resulted in the secretion of IFN-γ by NK cells, which was dependent on IL-2 produced by influenza A-specific T cells (56). Similarly, co-culture of Plasmodium falciparum-infected RBCs with PBMCs of malaria-naïve donors led to IFN-γ secretion by NK cells that required not only CD4+ T cells but also CD8+ and γδ T cells (27). However, this may not operate in all settings. Indeed, NK cell activation was detected in the absence of T cells in RAG−/− animals infected with Listeria monocytogenes or Mycobacterium tuberculosis (51, 57), suggesting that in these infections, the mechanisms controlling the initiation of NK cell activation in vivo differ.

Our data could have important implications for the development of long-term immunity to L. major, which is still not achieved with the current vaccines available. Our results may be linked to recent studies that implicated IL-2 in the establishment of T cell memory (26, 58). Indeed, T cell-mediated NK cell activation may regulate the differentiation of T cells (59). We would then expect that deficiencies in IL-2 signaling pathways would have an impact on the outcome of the response. Interestingly, human genetic studies revealed an association of functional polymorphisms in the β-chain CD122 with visceral Leishmaniasis triggered by Leishmania donovani (60). Further work will be needed to demonstrate such a link.

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Disclosures
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