NK cell activation in visceral leishmaniasis requires TLR9, myeloid DCs, and IL-12, but is independent of plasmacytoid DCs

Ulrike Schleicher,1,2 Jan Liese,1 Ilka Knippertz,2 Claudia Kurzmann,1 Andrea Hesse,1,2 Antje Heit,3 Jens A.A. Fischer,4 Siegfried Weiss,5 Ulrich Kalinke,6 Stefanie Kunz,1 and Christian Bogdan1,2

Natural killer (NK) cells are sentinel components of the innate response to pathogens, but the cell types, pathogen recognition receptors, and cytokines required for their activation in vivo are poorly defined. Here, we investigated the role of plasmacytoid dendritic cells (pDCs), myeloid DCs (mDCs), Toll-like receptors (TLRs), and of NK cell stimulatory cytokines for the induction of an NK cell response to the protozoan parasite Leishmania infantum. In vitro, pDCs did not endocytose Leishmania promastigotes but nevertheless released interferon (IFN)-α/β and interleukin (IL)-12 in a TLR9-dependent manner. mDCs rapidly internalized Leishmania and, in the presence of TLR9, produced IL-12, but not IFN-α/β. Depletion of pDCs did not impair the activation of NK cells in L. infantum–infected mice. In contrast, L. infantum–induced NK cell cytotoxicity and IFN-γ production were abolished in mDC-depleted mice. The same phenotype was observed in TLR9−/− mice, which lacked IL-12 expression by mDCs, and in IL-12−/− mice, whereas IFN-α/β receptor−/− mice showed only a minor reduction of NK cell IFN-γ expression. This study provides the first direct evidence that mDCs are essential for eliciting NK cell cytotoxicity and IFN-γ release in vivo and demonstrates that TLR9, mDCs, and IL-12 are functionally linked to the activation of NK cells in visceral leishmaniasis.

NK cells are key components of the innate immune response to infectious pathogens (1, 2). Activated NK cells are an early source of IFN-γ and thereby contribute to the development of type 1 Th cells (3, 4). They support the maturation of DCs (5, 6) and can exhibit cytolytic activity against host cells infected with certain viruses, bacteria, or protozoa (7–9). The activation of NK cells is a multifactorial process that involves soluble factors as well as stimulatory cell surface receptors that are triggered during interaction with ligand-positive target cells (10). In vitro studies revealed that human or mouse DCs can activate resting NK cells via direct cell–cell contact or the release of cytokines (e.g., IFN-α/β, IL-2, IL-12, and IL-18; reference 6). Depletion of a subset of lymphoid DCs (CD8α+) by anti-CD8 antibody treatment, which is not selective for DCs, partially abrogated Fms-like tyrosine kinase 3 ligand (Flt3L)-induced and NK cell–mediated tumor regression and impaired the expansion of Ly49H+ NK cells during murine cytomegalovirus infection (11, 12), but in vivo evidence for DC-dependent regulation of NK cell IFN-γ expression and cytotoxicity has not yet been published.

More recent in vitro studies on human DC populations and NK cells suggested that in addition to conventional or myeloid DCs (mDCs; CD11c−) plasmacytoid DCs (pDCs) can also activate NK cells for cytolytic activity in a type I IFN-α/β-dependent manner (13, 14). Mouse pDCs express a unique selection of myeloid and lymphoid cell surface markers (CD11b−, CD11c+),...
CD11c\textsuperscript{int}, B220\textsuperscript{+}, Ly6C\textsuperscript{+}, Gr-1\textsuperscript{+}, CD62L\textsuperscript{+}, and CD45RA\textsuperscript{+}; reference 15). They release large amounts of IFN-α/β in vitro and in vivo in response to DNA or RNA viruses, bacterial DNA, or synthetic oligodeoxynucleotides (ODNs) with unmethylated CpG motifs (CpG ODN; reference 15), synthetic guanosine-uridine (GU)-rich single-stranded RNA (ssRNA; references 16 and 17), or purified or synthetic hemoglobin (18).

In each of these cases, the production of IFN-α/β was dependent on Toll-like receptor (TLR)7 or TLR9, which are typically expressed by mouse pDCs (19). Both TLRs signal through the adaptor molecule myeloid differentiation factor 88 (MyD88), which recruits further signaling molecules and finally leads to the activation of NF-κB or IFN regulatory factor (IRF)-7, followed by rapid IFN-α/β production and IFN-γ expression (20).

A protective immune response against intracellular protozoan parasites of the genus *Leishmania* is characterized by the induction and expansion of IFN-γ-producing CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, which activate macrophages for the expression of antileishmanial effector pathways such as inducible nitric oxide synthase (21, 22). In the mouse models of experimental cutaneous (e.g., *Leishmania major*) and visceral (e.g., *Leishmania donovani* and *Leishmania infantum*) leishmaniasis, NK cells were found to participate in the innate immune response and control of the parasites (23–31). Endogenous and exogenous IL-12 or IFN-α/β were shown to confer NK cell activation (25, 28–30, 32, 33) and/or protective immunity in these models (21, 25, 29, 34–37). Furthermore, in vitro stimulation assays with pro- or amastigote parasites and certain mouse DCs (38–40), ex vivo immunohistochemical or flow cytometry analyses of lymphatic tissues (41, 42), as well as DC transfer and vaccination studies (43) identified DCs as a source of IL-12 during *Leishmania* infection. However, it is unknown how *Leishmania* parasites are initially sensed by the immune system to trigger an innate NK cell response during the early phase of *Leishmania* infection and whether the activation of NK cells in vivo requires interaction with CD11c\textsuperscript{high} mDCs, CD11c\textsuperscript{int} pDCs, and/or mDC-/pDC-derived cytokines.

In this study, we investigated which DC population, pathogen recognition receptor, and cytokine is essential for the induction of NK cell cytotoxicity and IFN-γ production in visceral leishmaniasis. Our parallel analysis of pDCs and the induction of NK cell cytotoxicity and IFN-γ production of both cytokines was strictly dependent on TLR9. Unexpectedly, pDCs (and, to a large extent, IFN-α/β receptor [IFNAR] signaling) were dispensable for the innate NK cell response to *L. infantum* in vivo. Instead, the activation of splenic NK cells after *L. infantum* infection required CD11c\textsuperscript{high} mDCs, TLR9, and IL-12.

**RESULTS**

**Differential production of IFN-α/β and IL-12p40 by pDCs and mDCs in response to *Leishmania* promastigotes**

Although mDCs are well-known targets of *Leishmania*, the interaction of pDCs with *Leishmania* has not yet been studied. Therefore, we investigated whether pDCs are targets of *Leishmania* parasites. Flt3L-expanded BM cells of C57BL/6 mice were exposed to *Leishmania* promastigotes and analyzed for the expression of costimulatory surface molecules.

**Figure 1. IFN-α/β expression in pDCs versus mDCs.** Cells were stimulated with 1 μM CpG ODN 2216, 50 ng/ml poly(I:C), 200 ng/ml LPS, *L. infantum*, *L. major*, or *L. braziliensis* promastigotes (M0I = 3) ± anti-CD40 mAb (5 μg/ml). (A) IFN-α/β production of sorted C57BL/6 BM-pDCs (Flt3L-BM culture) or BM-mDCs (GM-CSF-BM culture) after stimulation for 48 h. Mean ± SEM of two experiments. (B) IFN-α/β and IL-12p40 mRNA expression of sorted C57BL/6 BM-pDCs after stimulation for 24 h as determined by real-time RT-PCR. Mean ± SD of the calculated relative expression of seven independent experiments. (C) IFN-α/β production of purified splenic pDCs or splenic mDCs of 129Sv mice stimulated in parallel. Mean ± SEM of two experiments. ▼, not detectable.
Next, we compared the cytokine expression of C57BL/6 BM-pDCs (CD11b+CD11c+CD62L- cells sorted from Flt3L-expanded BM cultures) and BM-mDCs (CD11b+CD11c+CD86low cells sorted from GM-CSF-expanded BM cultures) after stimulation with live Leishmania promastigotes (multiplicity of infection [MOI] = 3, unless stated otherwise). pDCs generated copious amounts of IFN-α/β as detected by bioassay (Fig. 1 A) or ELISA (Fig. S1 C). Among all Leishmania species tested (L. major, L. infantum, and Leishmania braziliensis), L. infantum induced the highest release of IFN-α/β protein in 48 of a total of 58 experiments, which frequently was only one order of magnitude lower than the amount of IFN-α/β elicited by HSV-1 virus or CpG ODN 2216 (Fig. 1 A and Fig. S1 B). Real-time RT-PCR revealed a 10–1,000-fold induction of the mRNA expression of IFN-α and, with the exception of IFN-α11 and IFN-α13, of all IFN-α subtypes tested (Fig. 1 B). mDCs, in contrast, produced strikingly less IFN-α/β in response to CpG ODN 2216 and virtually no IFN-α/β after exposure to Leishmania parasites (Fig. 1 A).

Leishmania promastigotes also activated purified splenic pDCs (CD11b+CD11c+Gr-1−) for the production of IFN-α/β, whereas no IFN-α/β was detectable in the culture supernatants of purified splenic mDCs (CD11c+MHCII+; Fig. 1 C). Poly(I:C), which targets TLR3, and LPS, which interacts with TLR4, were inactive on BM-pDCs and splenic pDCs but elicited an IFN-α/β response in BM-mDCs and splenic mDCs (Fig. 1, A and C, and not depicted).

BM-pDCs co-cultured with Leishmania promastigotes released TNF, but not MIP-2α or nitric oxide (not depicted). Both BM-pDCs and BM-mDCs produced IL-12p40 after stimulation with CpG ODN 2216 or Leishmania promastigotes. The CpG- or Leishmania-induced IL-12p40 release of mDCs was higher compared with pDCs and enhanced by anti-CD40 (see WT pDCs and mDCs in Fig. 2 D). IL-12p70 remained undetectable (not depicted).

These data demonstrate that Leishmania promastigotes potently activate mouse pDCs, but not mDCs, for the expression of IFN-α/β mRNA and protein, whereas both DC populations are triggered for the release of IL-12p40.

The induction of IFN-α/β and/or IL-12 in pDCs and mDCs by Leishmania requires TLR9

The data presented above raised the question of which pathogen recognition receptor(s) on the surface of pDCs and mDCs transmits the signal for the induction of IFN-α/β and/or IL-12 by Leishmania.

Mouse pDCs express a very limited spectrum of TLRs (TLR7 and TLR9), which all signal via the MyD88 adaptor molecule (19). When sorted WT and MyD88−/− BM-pDCs were exposed to Leishmania promastigotes or CpG ODN 2216, we found that both the CpG- and the Leishmania-induced IFN-α/β release were entirely dependent on MyD88 (Fig. 2 A). The IFN-α/β production in response to L. infantum or L. major was completely abolished in the absence of TLR9 in seven out of eight experiments (in one experiment, the IFN-α/β release was ~100 U/ml). After stimulation of TLR9−/− BM-pDCs with L. braziliensis, IFN-α/β was undetectable in three of eight experiments and reduced by >95% in the remaining experiments (Fig. 2 B). As expected, CpG ODN 2216 did not induce any IFN-α/β in TLR9−/− pDCs (Fig. 2 B), whereas synthetic GU-rich ssRNA, a TLR7 ligand (16, 17), clearly triggered the release of IFN-α/β (mean U/ml ± SEM of two experiments: 8,789 ± 3,096).

The production of IFN-α/β during viral infections or in response to certain TLR9 ligands is regulated by a positive feedback loop in which the early secreted IFN-β and IFN-α4 initiate further IFN-α/β expression via IFNAR-mediated de novo synthesis of IRF-7 (15, 20). As pDCs constitutively express high levels of IRF-7 and release huge amounts of IFN-α even in the absence of an autocrine feedback loop (15, 44), we tested whether the Leishmania-induced IFN-α/β production...
Leishmania-induced IFN-α/β production by pDCs does not require replication, stage maturation, viability, or uptake of the parasites and can be mimicked by Leishmania DNA

To characterize the parasite requirements for the induction of IFN-α/β, we exposed sorted C57BL/6 BM-pDCs to (a) viable promastigotes of the logarithmic or stationary growth phase; (b) viable, but irradiated (i.e., replication-deficient) promastigotes; (c) freeze-thaw lysates of stationary-phase Leishmania promastigotes; or to (d) boiled lysates of Leishmania promastigotes. In all these cases, the induction of IFN-α/β was in the same order of magnitude (Fig. 3 A, top, and not depicted). This finding suggested that Leishmania DNA at least partially accounts for the stimulatory activity of whole parasites.

Eukaryotic DNA contains unmethylated CpG-DNA motifs, which might cause TLR9-dependent stimulation of immune cells (45–47). When C57BL/6 BM-pDCs were stimulated with 0.5–5 μg/ml genomic DNA (gDNA) from L. infantum promastigotes, the production of IFN-α/β was similar to that after co-culture with viable L. infantum parasites at an MOI of 3 (Fig. 3 A, bottom). At low concentrations (0.1 μg/ml), L. infantum gDNA induced only small (<300 U/ml; six experiments) or undetectable quantities of IFN-α/β (eight experiments; Fig. 3 A, bottom), whereas the equivalent number of whole parasites (MOI = 1.2, i.e., corresponding to 0.1 μg/ml L. infantum gDNA) still potently induced IFN-α/β (Fig. S1 B).

No IFN-α/β was measurable when TLR9−/− pDCs were stimulated with L. infantum gDNA or when WT pDCs were exposed to L. infantum lysates treated with DNase, L. infantum gDNA treated with DNase, L. infantum mitochondrial kinetoplast DNA (kDNA), mouse splenic gDNA, human blood gDNA, or to apoptotic (UV-irradiated or peroxynitrite-treated) annexin V+ pDCs. The DNase used did not exert nonspecific inhibitory effects because the IFN-α/β-inducing activity of ssRNA remained unaltered (Fig. 3 A, top and bottom, and not depicted). Thus, the TLR9-dependent IFN-α/β release is parasite gDNA specific and is not due to the recognition of self-DNA.

As TLR9 is expressed in endosomal and lysosomal compartments and requires an acidic pH to interact with ODN or DNA ligands (48), we tested whether chloroquine, which prevents endosomal acidification, can inhibit Leishmania-induced IFN-α/β release. Both the CpG ODN– and the Leishmania-induced IFN-α/β production were blocked by chloroquine without detectable toxicity as assessed by trypanblue exclusion (not depicted). This finding suggested that Leishmania...
promastigotes might enter an acidic endosomal compartment of pDCs. However, multicolor fluorescent microscopy of sorted C57BL/6 pDCs (CD11b<sup>−</sup>CD11c<sup>−</sup>CD62L<sup>+</sup>), mDCs (CD11b<sup>+</sup>CD11c<sup>−</sup>), and BM-derived macrophages (BM-MΦ) revealed that mDCs and BM-MΦ, but not pDCs, internalize L. infantum promastigotes. Importantly, parasites were attached to the surface of ~10% of the pDCs (Fig. 3 B). When parasites and pDCs were separated by a transwell membrane, no induction of IFN-α/β was observed (64,049 and 64,618 U/ml vs. <1 U/ml in two experiments).

Collectively, these data indicate that the activation of pDCs by Leishmania promastigotes for the production of IFN-α/β requires cell–cell contact, but not uptake of the parasites. Leishmania gDNA is a potent inducer of IFN-α/β and is likely to contribute to the stimulatory activity of intact parasites.

The NK cell response to L. infantum in vivo is weakly impaired in IFNAR<sup>−/-</sup> mice and unaffected in pDC-depleted mice

The strong activation of pDCs for the release of IFN-α/β by Leishmania promastigotes in vitro led us to investigate whether this process also occurs in vivo and is relevant for the initiation of the NK cell response to the parasite. We used a model of visceral (i.e., hepatic, splenic, and BM) leishmaniasis (49) because BM-derived and splenic pDCs were highly responsive to the viscerotropic strain of L. infantum and therefore might also sense the parasite in vivo.

In C57BL/6 WT mice, i.v. infection of L. infantum led to a striking induction of NK cell cytotoxic activity that was maintained in IFNAR<sup>−/-</sup> mice (Fig. 4 A). In the spleen, L. infantum caused an increased IFN-γ mRNA expression within 8 h of infection (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20061293/DC1) and the production of IFN-γ protein by NK cells. The percentage of IFN-γ<sup>+</sup> cells within the NK1.1<sup>+</sup>CD3<sup>−</sup> splenic NK cell population was similar in WT and IFNAR<sup>−/-</sup> mice at the 12-h time point but was significantly reduced at 24 h of infection in the IFNAR<sup>−/-</sup> group (Fig. 4 B). These results suggest that the L. infantum–induced and IFNAR-dependent secretion of IFN-α/β by pDCs that we observed in vitro is only partially involved in the activation of NK cells and/or does not occur to the same extent in vivo. In line with the latter possibility, we consistently found only a very weak induction (approximately factor 3–4) of IFN-α/β mRNAs in L. infantum–infected C57BL/6 WT mice (Fig. S2; see also WT mice in Fig. S4 A and Fig. 8 C), which was comparable in IFNAR<sup>−/-</sup> mice (Fig. S2). In addition, IFN-α or IFN-β protein was not detectable by ELISA in the serum or plasma of various strains of WT mice (C57BL/6, 129Sv, and BALB/c) within 4–24 h after L. infantum infection (not depicted).

To directly address the role of pDCs, we injected the pDC-specific anti–pDC-1 mAb twice before infection, which led to an ~80–90% reduction of the Siglec-H<sup>−</sup>CD11c<sup>−</sup>CD11b<sup>+</sup> splenic pDCs at all time points of infection tested (not depicted). This depletion protocol completely blocked the pDC-dependent IFN-α production in mice (50) elicited by i.v. injection of CpG ODN (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20061293/DC1).

In contrast, control IgG–treated or pDC-depleted C57BL/6 or 129Sv mice infected with L. infantum showed comparable levels of NK cell cytotoxicity, IFN-γ production, and splenic IFN-α4 and IFN-β mRNA expression (Fig. 4, C and D, and not depicted). Thus, pDCs are unlikely to control the early NK cell response to L. infantum.

The innate NK cell response to L. infantum requires IL-12 and CD11c<sup>+</sup> DCs

Because IFN-α/β had only a limited impact on the NK cell activation during the early phase of L. infantum infection, we tested whether IL-12 controls the onset of the NK cell response in visceral leishmaniasis. i.v. infection with L. infantum led to strong NK cell cytotoxicity and IFN-γ expression in WT mice, but not in IL-12p35<sup>/−</sup>/p40<sup>/−</sup> (devoid of IL-12 and IL-23) or IL-12p35<sup>/−</sup>/p40<sup>/−</sup> mice (devoid of IL-12; Fig. 5, A–D). Thus, IL-12 is essential for the activation of NK cells in L. infantum–infected mice. Given that mDCs were more potent producers of IL-12 than pDCs (notably after cross-linking of CD40; Fig. 2 D) and that the depletion of pDCs did not prevent the L. infantum–induced NK cell response (Fig. 4, C and D), we postulated that the IL-12–dependent NK cell activation in vivo is driven by mDCs.
To investigate this directly, we used CD11c-diphtheria toxin (DT) receptor (DTR)/GFP mice that carry a transgene encoding a fusion protein of DTR and GFP under control of the promoter of the mouse CD11c gene (51). 2 d after injection of DT, CD11c<sup>high</sup>MHCII<sup>+</sup>CD11b<sup>+</sup> splenic mDCs were ablated, whereas pDCs and all other CD11c<sup>int</sup> cells remained unaffected (Fig. 6 A). In agreement with a previous report (52), we observed that the DT treatment not only depleted CD11c<sup>high</sup>MHCII<sup>+</sup> mDCs, but also MOMA-1<sup>+</sup> metallophilic macrophages (MMs) and ERTR-9<sup>+</sup> marginal zone macrophages (MZMs) in the spleen (Fig. 6 B). However, 5 d after DT treatment, CD11c<sup>high</sup>MHCII<sup>+</sup> mDCs had repopulated the spleen to a large, albeit varying extent in individually analyzed mice (Fig. 6 C), whereas ERTR-9<sup>+</sup> MZMs remained completely depleted, and only very few MOMA-1<sup>+</sup> MMs became visible in all 10 individually analyzed mice (Fig. 6 D). F4/80<sup>+</sup> red pulp macrophages were not significantly affected by the DT treatment in CD11c-DTR/GFP mice (51, 52; not depicted), and the percentage of CD11b<sup>+</sup> cells even increased at days 2 and 5 after DT treatment (Fig. 6 A and not depicted).

When WT and CD11c-DTR/GFP mice were treated with DT, infected with L. infantum 24 h later, and analyzed 12 or 24 h after infection (i.e., at day 2 after DT), up-regulation of IFN-γ mRNA in the spleen, activation of NK cells, and IL-12p40 production of mDCs were seen in infected WT mice, but not in the transgenic mice (Fig. 7, A and B, and Fig. S4, A and B, which is available at http://www.jem.org/cgi/content/full/jem.20061293/DC1). Importantly, the number of splenic NK cells (CD3<sup>−</sup>NK1.1<sup>+</sup>) and their expression of IFN-γ after in vitro activation with the DC-independent stimuli PMA/ionomycin was comparable in DT-treated naive or infected WT and CD11c-DTR/GFP mice, except for a more prominent IFN-γ production in the case of infected WT mice, which reflects the prior DC-dependent priming of NK cells by L. infantum in vivo (Fig. S5, A and B). The L. infantum–induced up-regulation of IFN-α4, IFN-α5, and IFN-β mRNA at day 2 after DT was ~1,000-fold higher in the spleen of CD11c<sup>high</sup> mDC-depleted mice than in the respective control mice (Fig. S4 A), which, however, did not rescue the NK cell response in those mice. At day 5 after DT, in contrast, NK cell cytotoxicity and IFN-γ production after L. infantum infection were clearly restored in CD11c-DTR/GFP mice (Fig. 7, C and D).

Considering the varying degree of restoration of NK cell activation, the spleens of 10 CD11c-DTR/GFP mice were split and analyzed in parallel by FACS, immunohistology,
compared with WT PBS and CD11c-DTR staining of NK cells (CD3 at day 2 after DT. Mean of NK cells during the innate phase of requires IL-12 and the presence of CD11c high DCs, but not

JEM VOL. 204, April 16, 2007

The critical role of mDCs and IL-12 for the activation of NK cells in L. infantum–infected mice

TLR9 is required for the production of IL-12 and the activation of NK cells in L. infantum–infected mice

and NK cell cytotoxic assays, which revealed that the magnitude of NK cell cytotoxicity solely correlated with the percentage of reconstitution of CD11c<sup>high</sup>MHCII<sup>+</sup> mDCs (r<sup>2</sup> = 0.77; Fig. S6). MZMs and MMs were uniformly absent and the distribution and expression level of CD11b<sup>+</sup> or F4/80<sup>+</sup> in the red pulp were comparable in all mice tested (not depicted). A similar correlation was seen between NK cell IFN-γ production and the reconstitution of CD11c<sup>high</sup>MHCII<sup>+</sup> mDCs (r<sup>2</sup> = 0.66; not depicted). We conclude that activation of NK cells during the innate phase of L. infantum infection requires IL-12 and the presence of CD11c<sup>high</sup> DCs, but not of MZMs or MMs.

TLR9 is required for the production of IL-12 and the activation of NK cells in L. infantum–infected mice

whether TLR9 is essential for the expression of IL-12 and the initiation of the NK cell response in vivo. As observed before, i.v. infection of WT mice with L. infantum promastigotes was followed by a rapid induction of NK cell cytotoxicity and IFN-γ protein expression in the spleen. In TLR9<sup>−/−</sup> mice, in contrast, no NK cell activation was measurable in response to L. infantum (Fig. 8, A and B). Both the number of NK cells as well as the activation of NK cells by the TLR3 ligand poly(I:C) remained unaltered, indicating that the deletion of TLR9 does not lead to the depletion or to a universal functional suppression of NK cells (Fig. 8 B and not depicted).

The difference in the IFN-γ expression between WT and TLR9<sup>−/−</sup> mice during the innate phase of L. infantum infection was also seen by quantitative RT-PCR analysis of RNA samples prepared from total splenic tissue. In accordance with the TLR9-dependent production of type I IFNs and IL-12p40 by pDCs and/or mDCs in vitro, the L. infantum–induced expression of IFN-α4, IFN-β, and IL-12p40 mRNA was reduced in TLR9<sup>−/−</sup> compared with WT mice. However, due to the cell type–restricted expression and up-regulation of type I IFNs and IL-12, the factors by which the mRNA levels of these cytokines were increased in the spleen of WT mice after infection were not high enough to allow for significant differences between WT and TLR9<sup>−/−</sup> mice for all genes, at all time points of infection, and in each individual experiment (Fig. 8 C and not depicted). To definitively answer the question of TLR9-dependent expression of IL-12 in vivo, we resorted to single cell analyses using intracellular cytokine staining. In the spleen of L. infantum–infected mice, IL-12p40 protein was exclusively found in CD11c<sup>high</sup> mDCs (∼4% of all living spleen cells; see Fig. 6 A), but not in F4/80<sup>+</sup> macrophages (not depicted). As shown in Fig. 8 D, the L. infantum–induced production of IL-12p40 by CD11c<sup>high</sup> mDCs in the spleen and its accumulation in the plasma of WT mice were absent in TLR9<sup>−/−</sup> mice. IL-12p70 was not detectable in the plasma samples (not depicted).

Collectively, these data show that TLR9 is strictly required for the activation of NK cells in visceral leishmaniasis because it is essential for the production of IL-12 by CD-11c<sup>high</sup> mDCs.

DISCUSSION

In the past, only few studies had analyzed the role of NK cells in visceral leishmaniasis. In untreated Indian patients with visceral leishmaniasis, the cytolytic activity of peripheral blood NK cells was reduced (53). In experimental visceral leishmaniasis, NK cell–deficient beige mice (bg/bg) failed to eliminate L. donovani (23). After transfer of syngeneic cloned NK cells into bg/bg mice, the splenic parasite burdens were indistinguishable from those of normal WT mice (24). Finally, a 7-d treatment of BALB/c mice with IL-12 led to a 70% reduction of the liver parasite load compared with untreated control mice, whereas in IL-12–treated but NK cell–depleted mice, the decrease of the parasite numbers was only 30% (25). Collectively, all these earlier observations argued for a protective function of NK cells during primary visceral leishmaniasis.
However, neither the process of innate NK cell activation nor the underlying mechanisms have been investigated in the past.

To the best of our knowledge, this study is the first to demonstrate that the activation of NK cell functions in vivo requires the presence of mDCs (but not pDCs). Previous analyses in tumor or viral models were based on the application of anti-CD8 antibodies (which deplete CD8α+ lymphoid cells without being selective for these cells) and/or did not directly investigate the effector functions of NK cells (cytotoxicity and IFN-γ production; references 11, 12, and 54).

Another novelty of our report is the strict requirement of TLR9 for the induction of an NK cell response to a nonviral pathogen. This is particularly surprising, because unlike DNA viruses that were shown to drive NK cell activation in a TLR9-dependent manner (55, 56), Leishmania parasites are complex, eukaryotic pathogens that express a wide variety of possible ligands for pattern recognition receptors. Our results show that the early NK cell response to a visceral infection with L. infantum is completely dependent on TLR9, CD11chigh mDCs, and IL-12, weakly impaired in the absence of IFN-α/β, and unaffected after depletion of pDCs.

Therefore, this study offers a coherent picture of the receptor, cellular, and cytokine requirements of NK cell activation in vivo. Finally, the essential role of TLR9 for innate NK cell activation observed here might serve as a plausible explanation for the previously reported TLR9 dependency of adaptive Th1 immune responses in other microbial infection models (45, 46, 57).

pDCs, IFN-α/β, and TLR-9-dependent NK cell activation

Exposure of pDCs to promastigotes of different Leishmania species led to a rapid induction of IFN-β and a broad spectrum of IFN-α isoforms. The IFN-α/β release was not dependent on the parasite stage, replication, viability, integrity, or uptake but required a direct contact between the pDCs and the promastigotes, and an acidification of endosomal/lysosomal compartments that are thought to carry the TLR9 receptor (19, 48). Genomic, but not the evolutionary ancient mitochondrial (kinetoplast) DNA of Leishmania parasites, was sufficient to mimic the TLR9-dependent induction of IFN-α/β by intact promastigotes. At first glance, the TLR9-dependent and chloroquin-inhibitable stimulation of pDCs by intact promastigotes is difficult to reconcile with the
absence of detectable parasite uptake in pDC/Leishmania cocultures. However, as extrusion of gDNA by viable eukaryotic cells has been observed (58), and DNase treatment significantly (∼75%) reduced the production of IFN-α/β by pDCs exposed to Leishmania, our results are compatible with the idea that gDNA released by promastigotes during their attachment to pDCs accounts for the strong induction of IFN-α/β. We wish to point out that our findings do not exclude the possibility that another, so far unknown, heat-resistant component of Leishmania functions as TLR9 ligand and contributes to the activation of pDCs to produce IFN-α/β by intact parasites. In this respect, it is noteworthy that at least one non-DNA ligand of TLR9, i.e., hemozoin (a degradation product of host cell–derived heme in Plasmodium-infected erythrocytes), has been described that triggered human, but not mouse, pDCs for the release of IFN-α/β (18, 59).

In light of the strong production of IFN-α/β by pDCs exposed to L. infantum in vitro, the observation that a 90% reduction of the number of pDCs in the spleen did not affect the Leishmania-induced NK cell response was unexpected. As there was only a limited up-regulation of type I IFN mRNAs in the infected spleen that was not altered by the depletion of pDCs, splenic pDCs might not be targeted and/or activated by L. infantum in vivo during the early phase of infection. In this respect, visceral leishmaniasis differs from viral infection models in which pDC depletion resulted in a decrease of the expression of IFN-α (55, 60). However, even in viral infections pDCs are not necessarily required for NK cell cytotoxic activity (55). The fact that pDCs were dispensable for the elicitation of an innate NK cell response in L. infantum–infected mice does not exclude that the interaction between pDCs and Leishmania might be relevant during later stages of visceral leishmaniasis.

Our analysis of IFNAR−/− mice revealed that IFN-α/β (possibly released by cells other than pDCs) contributes to the induction of NK cell IFN-γ expression, but not of NK cell cytotoxicity in L. infantum–infected mice. This contrasts with (a) the role of IFN-α/β in mouse cytomegalovirus–infected mice, in which IFN-α/β was required for the induction of NK cell cytotoxicity, but not IFN-γ expression (61); and (b) with the function of IFN-α/β in experimental cutaneous leishmaniasis, where anti–IFN-α/β drastically reduced both the NK cell cytotoxicity and the early IFN-γ peak in the draining lymph nodes of L. major–infected mice (28). pDCs have been detected in the lymph nodes of L. major–infected mice (62), but whether they are necessary for the innate NK cell response and account for the previously reported early IFN-α/β production in cutaneous leishmaniasis (28) has not yet been investigated.

CD11chigh mDCs, IL-12, and TLR9-dependent NK cell activation

CD11c-DTR/GFP transgenic mice (51) are currently the only mouse model to eliminate CD11chigh DCs. To the best of our knowledge, this is the first study in which these mice have been used to demonstrate that CD11chigh DCs are necessary for the priming of NK cell functions (IFN-γ production and cytotoxicity) in vivo. Recently, it was reported that DT treatment of CD11c-DTR/GFP mice, in addition to CD11chigh DCs, also depletes MZMs and MMs in the spleen (52), which we could confirm in both naive and L. infantum–infected mice (Fig. 6 and not depicted). Although viscerotropic Leishmania parasites were previously shown to selectively activate DCs, but not MZMs, of the spleen (41), we carefully addressed the potential role of MZMs and MMs for the activation of NK cells. The time course and functional analyses shown in Fig. 6, Fig. 7, and Fig. S6 demonstrated that in L. infantum–infected, DT-treated CD11c-DTR/GFP mice, NK cell activation was rapidly restored once CD11chigh DCs repopulated the spleen, although at these time points both MZMs and MMs were still absent. Thus, NK cell activation requires CD11chigh DCs, whereas MZMs and MMs are clearly dispensable. It is also unlikely that the absence of NK cell activation in L. infantum–infected, DT-treated CD11c-DTR/GFP mice results from a suppressive or toxic effect of DT or dead DCs on NK cells. First, DT treatment did not reduce the number of NK cells. Second, DT did not impair the response of NK cells to PMA/ionomycin (Fig. S5). Third, the function of NK cells was fully restored after the repopulation of CD11chigh DCs, which further excludes a cytotoxic or long-lasting suppressive effect of DT on splenic NK cells.

The TLR9-dependent induction of IL-12p40 by L. infantum in mDCs in vitro (Fig. 2 D) and in vivo (Fig. 8 D), the equally absent innate NK cell response to L. infantum in TLR9−/−, IL-12−/−, and CD11chigh-depleted mice (Figs. 5, 7, and 8), and the lack of infection-induced IL-12p40 expression in CD11chigh-depleted mice (Fig. S4 B) strongly argues for the following model of NK cell activation in which TLR9, mDCs, IL-12, and NK cells are coherently linked: L. infantum activates CD11chigh DCs via TLR9 for the generation of IL-12 that subsequently triggers NK cell cytotoxicity and IFN-γ production. The dominant role of mDCs and IL-12 is further underlined by the observation that the depletion of CD11chigh mDCs before L. infantum infection did not diminish the number of splenic pDCs (Fig. 6 A) and was accompanied by a roughly 1,000-fold increase of the IFN-α and IFN-β mRNA expression (Fig. S4 A), which, however, was insufficient to prevent the loss of NK cell activity. Our results also offer a mechanistic and functional explanation for the close and prolonged interactions between NK cells and mDCs that were recently seen in the lymph nodes of mice infected with L. major (experimental cutaneous leishmaniasis) using intravital microscopy (31).

Human NK cells were previously shown to express TLRs, including TLR2, TLR3, and TLR9 (63–65). This led us to consider whether L. infantum might directly activate mouse NK cells in a TLR9-dependent manner. Previous and present observations strongly argue against this possibility. First, FACS–sorted, IL-2–expanded mouse splenic NK1.1+CD3− NK cells did not express TLR9 mRNA as assessed by RT-PCR (unpublished data). Second, FACS–sorted,
IL–2–expanded or MACS-sorted (DX5⁺) naïve splenic NK cells were neither activated by CpG ODN (55) nor by Leishmania promastigotes (unpublished data) for the expression of IFN-γ or cytolytic activity. Third, in the absence of CD11c<sub>high</sub> DCs or IL–12, no NK cell activity was detectable in *L. infantum*-infected mice.

In summary, this study revealed an unexpected, dominant role of TLR9 for the initiation of the NK cell response to a complex eukaryotic pathogen. Although initial in vitro experiments suggested that both pDCs and mDCs are valid candidates to deliver activating signals to NK cells, our in vivo analyses demonstrated that TLR9, CD11c<sub>high</sub> mDCs, and IL–12, but not pDCs and type I IFNs, were essential for NK cell cytotoxicity and IFN-γ production in visceral leishmaniasis. The observed TLR9- and IL–12-dependent NK cell activation by mDCs suggests a certain hierarchy within the receptors, cell types, and cytokines previously shown to exert activating effects on NK cells, which might also hold true for infections with other intracellular pathogens.

### MATERIALS AND METHODS

**Mice, parasites, and infection**

Female C57BL/6, BALB/c, and 129Sv (PasIco) mice were from The Jackson Laboratory. Breeding pairs of IL-12p35<sup>-/-</sup> mice (fifth generation backcross to BALB/c; reference 34) and C57BL/6; BALB/c, and 129Sv (PasIco) mice were from Charles River Laboratories, and CD11c-DTR/GFP transgenic mice (15th generation backcross to BALB/c; reference 29) were from T. Stamminger, University of Erlangen, Erlangen, Germany).

For infection, mice were injected i.v. in the retro-orbital vein or in the tail vein with 300 μl PBS or 10⁶ stationary phase *L. infantum* promastigotes or with GU-rich antigen (freeze-thaw lysates of promastigotes; MOI = 3:1), or iNOS<sup>-/-</sup> mice (68), or 50 μg/ml poly(I:C) (Sigma-Aldrich), 50 ng/ml poly(I,C) (Sigma-Aldrich), 200 ng/ml LPS (*Escherichia coli* O111:B4; Sigma-Aldrich), 50 μg/ml mGM-CSF (75). GM-CSF–expanded BM cultures (day 8) contained ≥80% CD11b<sup>+</sup>CD11c<sup>+</sup> mDCs, which were further purified as immature CD11c<sup>+</sup>CD86<sup>+</sup>CD80<sup>+</sup> mDCs by MoFlo sorting (purity ≥96%).

**In vivo treatment**

To deplete pDCs, mice were i.p. injected with 500 μg rat anti–pDC-1 mAb or control rat IgG (The Jackson Laboratory) at 24 and 4 h before i.v. injection of *L. infantum*, 5 μg of a phosphorothioate-modified CpG ODN (50), or PBS. The CpG ODN was mixed with 30 μl of the cationic liposome preparation DOTAP (Roche Diagnostics) in a volume of 300 μl PBS (50). 4, 6, 8, 12, and 24 h after injection, spleen cells were analyzed for the presence of CD11b<sup>+</sup>CD11c<sup>+</sup>Siglec-H<sup>+</sup> cells to control depletion. To deplete mDCs, CD11c<sup>-DTR</sup>/GFP mice received an i.p. injection of DT (4 ng/g body weight; Sigma-Aldrich) 1 or 4 d before *L. infantum* injection. The reduction of CD11c<sup>+</sup>MHCII<sup>+</sup> mDCs was controlled by FACS analyses.

**FACS analysis**

For surface phenotyping and cell sorting, the following unconjugated, fluorochrome (FITC-, PE-, or APC-)–labeled biotinylated mAbs were used (all from BD Biosciences unless otherwise stated): anti-CD11b (M1/70), anti-Ly6C (ER–MP20; BMA Biomedicals), anti-Ly6G (Gr-1), anti-CD62L (MEL-14), anti-CD11c (HL3), anti-CD45R/B220 (RA3-6B2), anti-CD11b (M1/70), anti-CD80 (3/23), anti-CD86 (16-10A1), anti-CD186 (GL1), anti-NK1.1 (PK136), anti-CD49b (DX5), anti-CD3 (145-2C11), anti-mPDCA-1 (Miltenyi Biotec), and anti-Siglec-H (440c; Hycult Biotechnology).

In vivo treatment demonstrated that TLR9, CD11c<sub>high</sub> mDCs, and IL–12, but not pDCs and type I IFNs, were essential for NK cell cytotoxicity and IFN-γ production in visceral leishmaniasis. The observed TLR9- and IL–12-dependent NK cell activation by mDCs suggests a certain hierarchy within the receptors, cell types, and cytokines previously shown to exert activating effects on NK cells, which might also hold true for infections with other intracellular pathogens.
K digestion of promastigotes, followed by phenol/chloroform extraction and ethanol precipitation or by using the Blood&Cell Culture DNA kit (QIAGEN). In both cases, RNA was removed with DNase-free RNaseA (Invitrogen). LDNA was prepared as described previously (76) and controlled by gel electrophoresis.

Cytokine and nitrite measurements

IFN-α/β levels were determined with a L929/vesicular stomatitis virus protection assay using triplicates and serial twofold dilutions of the culture supernatants (28). Purified mouse IFN-α/β and a neutralizing sheep anti-IFN-α/β antiserum (provided by I. Gresser, Institut Curie, Paris, France) was used as a standard to ascertain that all antiviral activity in supernatants was due to IFN-α/β. The content of IFN-α (including IFN-α1, IFN-α4, IFN-α5, and IFN-α9) or IFN-β (both from PBL Biomedical Laboratories), TNF (sensitivity 40 pg/ml; R&D Systems), MIP-2 (sensitivity 20 pg/ml; Nordic Biosite), IL-12p40, or IL-12p70 (sensitivity 40 pg/ml; BD Biosciences) was measured by ELISA. NO2− was determined by the Griess assay (77).

Intracellular cytokine staining

IFN-γ staining in NK cells. Spleen cells of infected or control mice were restimulated for 6 h in the presence of 10 μg/ml brefeldin A with medium alone or with YAC-1 tumor target cells (ratio 1:1) for repeated priming of the NK cells or with 50 ng/ml PMA (Sigma-Aldrich) and 750 ng/ml ionomycin (Sigma-Aldrich). After staining of NK cell surface markers (CD3-NK1.1+ or CD3-DSX5+), the cells were fixed with CytopermCytofix (BD Biosciences) and incubated with APC-conjugated rat anti-mouse IFN-γ (XMG1.2; BD Biosciences) as described previously (77).

IL-12p40 staining in CD11c+ cells. Applying the same method as described above, spleen cells were restimulated with medium alone or, as a positive control, with 1 μM CpG ODN 1668 (Thermo Electron). For surface staining, anti-CD11c, anti-CD11b, and anti-F4/80 (CI:A3-1; Serotec) mAbs were used. IL-12p40 was stained by an APC-conjugated rat anti-mouse IL-12p40/p70 mAb (C15.6; BD Biosciences).

NK cell cytotoxicity

After determining the percentage of NK1.1+CD3− or DX5+CD3− NK cells within whole spleen cells, the NK cell cytotoxicity against YAC-1 tumor cells was determined (27, 28).

Immunofluorescence microscopy

Purified BM-pDCs and BM-mDCs or BM-MΦ (77) were incubated with Leishmania promastigotes for 16 h. Thereafter, nonadherent pDCs and mDCs were transferred to adhesion slides (MARIENFELD) and permeabilized in 1% saponin using human anti–L. major (provided by M. Colonna, Center 620 “Immunodeficiency” of the German Research Foundation (project A9), and P. Aichele and A. Diefenbach (Institute of Medical Microbiology and Immunology, Freiburg), and by the European Community (QLK2-CT-2001-02103). The authors have no conflicting financial interests.

Kolte et al.

and by a red alkaline phosphatase substrate (Vector Laboratories). Sections were counterstained with Meyer’s hemalum, mounted with Aquatex (Merck), and analyzed by light microscopy (Axioskop 2 plus; Carl Zeiss Microimaging, Inc.).

Rift Valley fever virus has been shown to enter macrophages for a limited time and to exit as virus-containing vesicles (56). Therefore, the time of infection with Rift Valley fever virus was optimized. The results of this experiment are shown in Fig. 5 A. Fig. 5 B shows the infection of macrophages with Rift Valley fever virus, followed by staining of infected macrophages with 10 μg/ml of IFN-γ (BD Biosciences) or IFN-γ-containing IFN-γ and IL-12p40 staining in CD11c+ cells. The following cycle conditions: 2 min at 50 °C, 10 min at 95 °C, and then 15 s at 95 °C and 60 s at 60 °C for 40 cycles. mRNA levels were calculated with the SDS 2.1 software (Applied Biosystems). The amount of mRNA of each gene was normalized to the housekeeping gene mHPRT-1. mRNA expression levels were calculated as the n-fold difference relative to the housekeeping gene by the formula: relative expression = 2−ΔΔCt[mean−control].

Statistics

Statistical analysis was performed using the unpaired Student’s t test.

Online supplemental material

Fig. S1 shows the up-regulation of costimulatory molecules and the dose-dependent induction of IFN-α/β (determined by bioassay or ELISA) in pDCs upon exposure to Leishmania promastigotes. Fig. S2 presents quantitative RT-PCR data on the expression of IFN-α/β and IFN-γ in C57Bl/6 WT and IFNAR−/− mice infected with 107 L. infantum. Fig. S3 illustrates the reduction of IFN-α production in mice challenged with CpG after prior depletion of pDCs. Fig. S4 shows the splenic expression of IFN-γ mRNA and type I IFN mRNAs as well as the expression of IL-12p40 protein in splenic DCs in DT-treated C57Bl/6 WT and CD11c-DTR/GFP transgenic mice after injection of DT and infection with L. infantum. Fig. S6 documents the linear correlation between NK cell cytotoxicity and DC reconstitution in the spleens of C57Bl/6 WT and CD11c-DTR/GFP transgenic mice. Figs. S1–S6 are available at http://www.jem.org/cgi/content/full/jem.20061293/DC1.

We are grateful to G. Alber (University of Leipzig, Germany), S. Bauer (University of Marburg, Germany), D. Busch (University of Munich, Germany), M. Colonna (Washington University, St. Louis), I. Gresser (Institut Curie, Paris), A. Krug (University of Munich, Germany), T. Stamminger (University of Erlangen, Germany), and P. Aichele and A. Diefenbach (Institute of Medical Microbiology and Hygiene, Freiburg) for advice or the generous supply of mice, reagents, or protocols.

This work was supported by the priority program "Innate Immunity of the German Research Foundation (Bo 9963/1-1, 3-2, 3-3), by the Collaborative Research Center 620 “Immunodeficiency” of the German Research Foundation (project A9), and by the European Community (QLK2-CT-2001-02103). The authors have no conflicting financial interests.

Fig. 1 S1 shows the up-regulation of costimulatory molecules and the dose-dependent induction of IFN-α/β (determined by bioassay or ELISA) in pDCs upon exposure to Leishmania promastigotes. Fig. S2 presents quantitative RT-PCR data on the expression of IFN-α/β and IFN-γ in C57Bl/6 WT and IFNAR−/− mice infected with 107 L. infantum. Fig. S3 illustrates the reduction of IFN-α production in mice challenged with CpG after prior depletion of pDCs. Fig. S4 shows the splenic expression of IFN-γ mRNA and type I IFN mRNAs as well as the expression of IL-12p40 protein in splenic DCs in DT-treated C57Bl/6 WT and CD11c-DTR/GFP transgenic mice after injection of DT and infection with L. infantum. Fig. S6 documents the linear correlation between NK cell cytotoxicity and DC reconstitution in the spleens of C57Bl/6 WT and CD11c-DTR/GFP transgenic mice. Figs. S1–S6 are available at http://www.jem.org/cgi/content/full/jem.20061293/DC1.

We are grateful to G. Alber (University of Leipzig, Germany), S. Bauer (University of Marburg, Germany), D. Busch (University of Munich, Germany), M. Colonna (Washington University, St. Louis), I. Gresser (Institut Curie, Paris), A. Krug (University of Munich, Germany), T. Stamminger (University of Erlangen, Germany), and P. Aichele and A. Diefenbach (Institute of Medical Microbiology and Hygiene, Freiburg) for advice or the generous supply of mice, reagents, or protocols.

This work was supported by the priority program "Innate Immunity of the German Research Foundation (Bo 9963/1-1, 3-2, 3-3), by the Collaborative Research Center 620 “Immunodeficiency” of the German Research Foundation (project A9), and by the European Community (QLK2-CT-2001-02103). The authors have no conflicting financial interests.
REFERENCES

1. French, A.R., and W.M. Yokoyama. 2003. Natural killer cells and viral infections. Curr. Opin. Immunol. 15:45–51.
2. Korbel, D.S., O.C. Finney, and E.M. Riley. 2004. Natural killer cells and innate immunity to protozoan pathogens. Int. J. Parasitol. 34:1517–1528.
3. Martin-Fontecha, A., L.L. Thomsen, S. Brett, C. Gerard, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2004. Induced recruitment of NK cells to lymph nodes provides IFN-γ for Th1 priming. Nat. Immunol. 5:1260–1265.
4. Laouar, Y., F.S. Sutterwala, L. Gorelik, and R.A. Flavell. 2005. Transforming growth factor-β controls T helper type 1 cell development through regulation of natural killer cell interferon-γ. Nat. Immunol. 6:600–607.
5. Moretta, A. 2002. Natural killer cells and dendritic cells: rendezvous in abused tissues. Nat. Rev. Immunol. 2:957–963.
6. Degli-Esposti, M.A., and M.J. Smyth. 2005. Close encounters of different kinds: dendritic cells and NK cells take centre stage. Nat. Rev. Immunol. 5:112–124.
7. Arase, H., and L.L. Lanier. 2004. Specific recognition of virus-infected cells by paired NK receptors. Rev. Med. Virol. 14:83–93.
8. Vankayalapati, R., A. Garg, A. Pogored, D.E. Griffith, P. Klucar, H. Safi, W.M. Girard, D. Cosman, T. Spies, and P.F. Barnes. 2005. Role of NK cell-activating receptors and their ligands in the lysis of mononuclear phagocytes infected with an intracellular bacterium. J. Immunol. 175:4611–4617.
9. Aranha, F.C.S., U. Ribeiro, P. Basé, C.E.P. Corbett, and M.D. Safi. 2003. Functional interactions between dendritic cells and NK cells during viral infection. J. Immunol. 167:7237–7243.
10. Carayannopoulos, L.N., and W.M. Yokoyama. 2004. Recognition of infected cells by natural killer cells. Curr. Opin. Immunol. 16:26–33.
11. Fernandez, N.C., A. Lozier, C. Flament, P. Ricciardi-Castagnoli, D. Bellet, M. Suter, M. Perricaudet, T. Tursz, E. Maraskovsky, and L. Zitvogel. 1999. Dendritic cells directly trigger NK cell function: crosstalk relevant in innate anti-tumor immune responses in vivo. Nat. Med. 5:405–411.
12. Andrews, D.M., A.A. Scalzo, W.M. Yokoyama, M.J. Smyth, and M.A. Degli-Esposti. 2003. Functional interactions between dendritic cells and NK cells during viral infection. Nat. Immunol. 4:175–181.
13. Gerosa, F., A. Gobbi, P. Zorzi, S. Burg, F. Briere, G. Carra, and F. Briere. 2004. Induced recruitment of NK cells to lymph nodes provides IFN-α/β and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. Immunity. 8:77–87.
14. Murray, H.W. 2004. The immunobiology of the TLR9 subfamily. J. Immunol. 173:1477–1497.
15. Murray, H.W. 1997. Endogenous interleukin-12 regulates acquired resistance in experimental visceral leishmaniasis. J. Immunol. 154:5320–5330.
16. Sacks, D., and N. Noben-Trauth. 2002. The immunology of susceptibility and resistance to Leishmania major in mice. Nat. Rev. Immunol. 2:845–858.
17. Bogdan, C. 2004. Reactive oxygen and reactive nitrogen metabolites as effector molecules against infectious pathogens. In The innate immune response to infection. S.H.E. Kaufmann, R. Medzhitov, and S. Gordon, editors. ASM Press, Washington, D.C. 357–396.
18. Kirkpatrick, C.E., and J.P. Farrell. 1982. Leishmaniasis in beige mice. Infect. Immun. 38:1208–1216.
19. Kirkpatrick, C.E., J.P. Farrell, J.F. Warner, and G. Deenert. 1985. Participation of natural killer cells in the recovery of mice from visceral leishmaniasis. Cell. Immunol. 92:163–171.
20. Murray, H.W., and J. Harpeshad. 1995. Interleukin 12 is effective treatment for an established systemic intracellular infection: experimental visceral leishmaniasis. J. Exp. Med. 181:387–391.
21. Scharton, T.M., and P. Scott. 1993. Natural killer cells are a source of IFN-γ that drives differentiation of CD4+ T cell subsets and induces early resistance to Leishmania major in mice. J. Exp. Med. 178:567–578.
22. Laskay, T., M. Röllinghoff, and W. Sollbach. 1993. Natural killer cells participate in the early defense against Leishmania major infection in mice. Eur. J. Immunol. 23:2237–2241.
23. Diefenbach, A., H. Schindler, N. Douhalser, E. Lorenz, T. Laskay, J. MacMicking, M. Röllinghoff, I. Greser, and C. Bogdan. 1998. Type 1 interferon (IFN-α/β) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. Immunity. 8:77–87.
24. Mattner, J., A. Wandera-Steenhäuser, A. Pahl, M. Röllinghoff, G.R. Majeau, P.S. Hochman, and C. Bogdan. 2004. Protection against progressive leishmaniasis by IFN-β. J. Immunol. 172:7574–7582.
25. Schleicher, U., J. Mattner, M. Blos, H. Schindler, M. Röllinghoff, M. Karaghiosoff, M. Müller, G. Werner-Felmayer, and C. Bogdan. 2004. Control of Leishmania major in the absence of Tyk2 kinase. Eur. J. Immunol. 34:519–529.
26. Scharton-Kersten, T., L.C.C. Afonso, M. Wysocka, G. Trinchieri, and P. Scott. 1995. IL-12 is required for natural killer cell activation and subsequent T helper 1 cell development in experimental leishmaniasis. J. Immunol. 154:5320–5330.
27. Laskay, T., A. Diefenbach, M. Röllinghoff, and W. Sollbach. 1995. Early passive containment is decisive for resistance to Leishmania major infection. Eur. J. Immunol. 25:2220–2227.
28. Mattner, J., M. Magram, J. Ferrante, P. Launois, K. Di Padova, R. Behin, M.K. Gately, J.A. Louis, and G. Alber. 1996. Genetically resistant mice lacking interleukin-12 are susceptible to infection with Leishmania major and mount a polarized Th2 cell response. Eur. J. Immunol. 26:1553–1559.
29. Sacks, D., and N. Noben-Trauth. 2002. The immunology of susceptibility and resistance to Leishmania major in mice. Nat. Rev. Immunol. 2:845–858.
30. Engwerda, C.R., M.L. Murphy, S.E.J. Cotterell, S.C. Smelt, and P.M. Kaye. 1998. Neutralization of IL-12 demonstrates the existence of discrete organ-specific phases in the control of Leishmania donovani. Eur. J. Immunol. 28:669–680.
31. Satozkar, A.R., S. Rodig, S.R. Telford, A.A. Satozkar, S.K. Ghosh, F. von Lichtenberg, and J.R. Davis. 2000. IL-12 gene-deficient C57BL/6 mice are susceptible to Leishmania donovani but have diminished hepatic immunopathology. Eur. J. Immunol. 30:834–839.
32. Koncny, P., A.J. Staag, H. Jebbari, N. English, R.N. Davidson, and S.C. Knight. 1999. Murine dendritic cells internalize Leishmania major promastigotes, produce IL-12 p40 and stimulate primary T cell proliferation in vitro. Eur. J. Immunol. 29:1803–1811.
33. von Stetten, E., Y. Belkaid, B.V. Nguyen, M. Cushing, D.L. Sacks, and M.C. Udey. 2000. Leishmania major-infected murine Langerhans cell-like dendritic cells from susceptible mice release IL-12 after infection and vaccinate against experimental cutaneous leishmaniasis. Eur. J. Immunol. 30:3498–3506.
IFN-α release and initiation of immune responses in vivo. J. Immunol. 176:6723–6732.

51. Mims, L.A., L.C. Menard, D.M. Fourneau, S. Darche, C. Ronet, D.W. Mielcarek, D. Buzonzi-Gatel, and L.H. Kasper. 2006. TLR9 is required for the gut-associated lymphoid tissue response following oral infection of Toxoplasma gondii. J. Immunol. 176:7589–7597.

52. Brinkmann, V., U. Reichard, C. Goossen, B. Fauler, Y. Uhrlemann, D.S. Weiss, Y. Weinrauch, and A. Zychlinsky. 2004. Neutrophil extracellular traps kill bacteria. Science. 303:1532–1535.

53. Pichyangkul, S., K. Yongvanitchit, U. Hum-arb, H. Hemmi, S. Akira, A.M. Krieg, O.G. Heppner, V.A. Stewart, H. Hasegawa, S. Lozaareunw, et al. 2004. Malana blood stage parasites activate human plasmacytoid dendritic cells and murine dendritic cells through a Toll-like receptor 9-dependent pathway. J. Immunol. 172:4926–4933.

54. Cervantes-Barragan, L., R. Züst, F. Weber, M. Spiegel, K.S. Lang, S. Akira, V. Thiel, and B. Ludewig. 2007. Control of coronavirus infection through plasmacytoid dendritic cell-derived type I interferon. Blood. 110:1131–1137.

55. Nguyen, K.B., T.P. Salazar-Mather, M.Y. Dalod, J.B. van Deussen, X.Q. Wei, F.Y. Liew, M.A. Caligiuri, J.E. Durbin, and C.A. Baron. 2002. Coordinated and distinct roles for IFN-α/β, IL-12, and IL-15 regulation of NK cell responses to viral infection. J. Immunol. 169:4297–4287.

56. Baldwin, T., S. Henri, J. Curtis, M. O’Keeffe, D. Vremec, K. Shortman, and E. Handman. 2004. Dendritic cell populations in Leishmania major-infected skin and draining lymph nodes. Infect. Immun. 72:1991–2001.

57. Hornung, V., S. Rothenfuss, S. Britsch, A. Krug, B. Jahrsdörfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of Toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J. Immunol. 168:4531–4537.

58. Becker, I., N. Salaiza, M. Aguirre, J. Delgado, N. Carrillo-Carrasco, L. Gutierrez-Kobeh, A. Ruiz, R. Cervantes, A. Perez Torres, N. Cabrera, et al. 2003. Leishmania lipophosphoglycan (LPG) activates NK cells through toll-like-receptor-2. Mol. Biochem. Parasitol. 130:65–74.

59. Sivori, S., M. Falco, M. Della Chiesa, S. Carlonmagno, M. Vitale, L. Moretta, and A. Moretta. 2004. CpG and double-stranded RNA trigger human NK cells through Toll-like receptor-3. Mol. Immunol. 40:1321–1328.

60. Muller, U., L. Steinhoff, F.L. Reis, S. Hemmi, J. Pavlović, R.M. Zinkernagel, and M. Aguet. 1994. Functional role of type I and type II interferons in antiviral defense. Science. 264:1918–1921.

61. Erlandsson, L., R. Blumenthal, M.-L. Eloranta, H. Engel, G. Alm, S. Weiss, and T. Landersted. 1998. Interferon-β is required for interferon-α production in mouse fibroblasts. Curr. Biol. 8:223–226.

62. Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakashima, and S. Akira. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity. 9:143–150.

63. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. Nature. 408:740–745.

64. Sollb förberg, K., A. Emmer, C. Bogdan, and M. Röllinghoff. 1986. Suppressive effect of cyclosporin A on the development of Leishmania tropica-induced lesions in genetically susceptible BALB/c mice. J. Immunol. 137:702–707.

65. Bogdan, C., G. Schönau, A.-L. Banuls, M. Hide, F. Pratlong, E. Lorenz, M. Röllinghoff, and R. Mertens. 2001. Visceral leishmaniasis in a German child that had never entered a known endemic area: case report and review of the literature. Clin. Infect. Dis. 32:302–307.

66. Sousa, A.C., M.E. Parise, M.M. Pompeu, J.M. Coelho Filho, I.A. Vasconcelos, J.W. Lima, E.G. Oliveira, A.W. Vasconcelos, J.R. David, and J.H. Maguire. 1995. Baboon leishmaniasis: a common manifestation of Leishmania (Viannia) braziliensis infection in Ceará, Brazil. Am. J. Trop. Med. Hyg. 53:380–385.

67. Gilliet, M., A. Boonstra, C. Patrule, S. Antonenko, X.L. Xu, G. Trinchieri, A. O’Gara, and Y.J. Liu. 2002. The development of murine plasmacytoid dendritic cell precursors is differentially regulated by...
FLT3-ligand and granulocyte/macrophage colony-stimulating factor. 

J. Exp. Med. 195:953–958.

74. Blasius, A., M. Celli, J. Maldonado, T. Takai, and M. Colonna. 2006. Siglec-H is an IPC-specific receptor that modulates type I IFN secretion through DAP12. Blood. 107:2474–2476.

75. Lutz, M.B., N. Kukutsch, A.L. Ogilvie, S. Rössner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J. Immunol. Methods. 223:77–92.

76. Shapiro, T.A., V.A. Klein, and P.T. Englund. 1999. Isolation of kinetoplast DNA. Methods Mol. Biol. 94:61–67.

77. Schleicher, U., A. Hese, and C. Bogdan. 2005. Minute numbers of contaminant CD8+ T cells or CD11b+CD11c+ NK cells are the source of IFN-γ in IL-12/IL-18-stimulated mouse macrophage populations. Blood. 105:1319–1328.

78. Stenger, S., N. Donhauser, H. Thüring, M. Röllinghoff, and C. Bogdan. 1996. Reactivation of latent leishmaniasis by inhibition of inducible nitric oxide synthase. J. Exp. Med. 183:1501–1514.