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

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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.

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Abbreviations used: BM-mDC, BM-derived myeloid DC; BM-MΦ, BM-derived macrophages; BM-pDC, BM-derived plasmacytoid DC; DT, diphtheria toxin; DTR, DT receptor; Flt3L, *fms*-like tyrosine kinase 3 ligand; gDNA, genomic DNA; GU, guanosine-uridine; IRF, IFN regulatory factor; IFNAR, IFN-α/β receptor; kDNA, kinetoplast DNA; mDC, myeloid DC; MM, metallophilic macrophage; MOI, multiplicity of infection; MyD88, myeloid differentiation factor 88; MZM, marginal zone macrophage; ODN, oligodeoxynucleotide; pDC, plasmacytoid DC; ssRNA, single-stranded RNA; TLR, Toll-like receptor.

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^{high}) 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^{-},

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The online version of this article contains supplemental material.

The Journal of Experimental Medicine

CD11c^{int}, B220$^+$, Ly6C$^+$, Gr-1$^+$, CD62L$^+$, and CD45RA$^+$; 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 hemozoin (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 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$^+$ and CD8$^+$ 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* infections. 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^{high} mDCs, CD11c^{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 mDCs revealed that only pDCs, but not mDCs, produced IFN-α/β after stimulation with *L. infantum* promastigotes, whereas pDCs as well as mDCs released IL-12. The *Leishmania*-induced 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^{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. *L. infantum* and *L. major* promastigotes up-regulated the expression of CD40, CD80, and CD86 on mPDCA-1$^+$CD11b$^-$CD11c$^+$ BM-derived pDCs (BM-pDCs; as well as on CD11b$^+$CD11c$^+$ BM-derived mDCs [BM-mDCs]) within the Flt3L culture, although to a lesser extent than the known pDC activator CpG ODN 2216 (Fig. S1 A, which is available at http://www.jem.org/cgi/content/full/jem.20061293/DC1, and not depicted; reference 19).

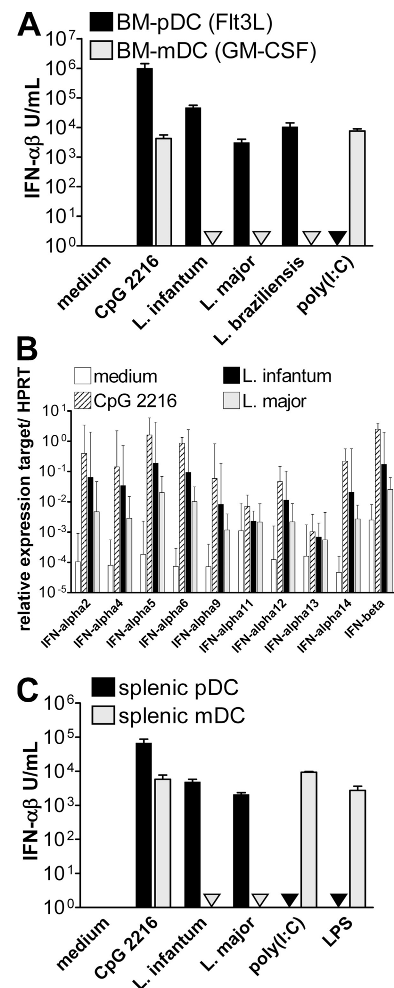

Figure 1. IFN-α/β expression in pDCs versus mDCs. Cells were stimulated with 1 μM CpG ODN 2216, 50 ng/ml poly(I:C), 200 ng/ml LPS, *L. infantum*, *L. major*, or *L. braziliensis* promastigotes (MOI = 3) ± anti-mCD40 mAb (5 μg/ml). (A) IFN-α/β production of sorted C57BL/6 BM-pDCs (Flt3L-BM culture) or BM-mDCs (GM-CSF-BM culture) after stimulation for 48 h. Mean ± SEM of two experiments. (B) IFN-α and IFN-β 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 ($CD11b^+$-$CD11c^+CD86^{low}$ 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-β 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 ($CD11b^-CD11c^{int}Gr\text{-}1^+$) for the production of IFN-α/β, whereas no IFN-α/β was detectable in the culture supernatants of purified splenic mDCs ($CD11c^{high}MHCII^+$; 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

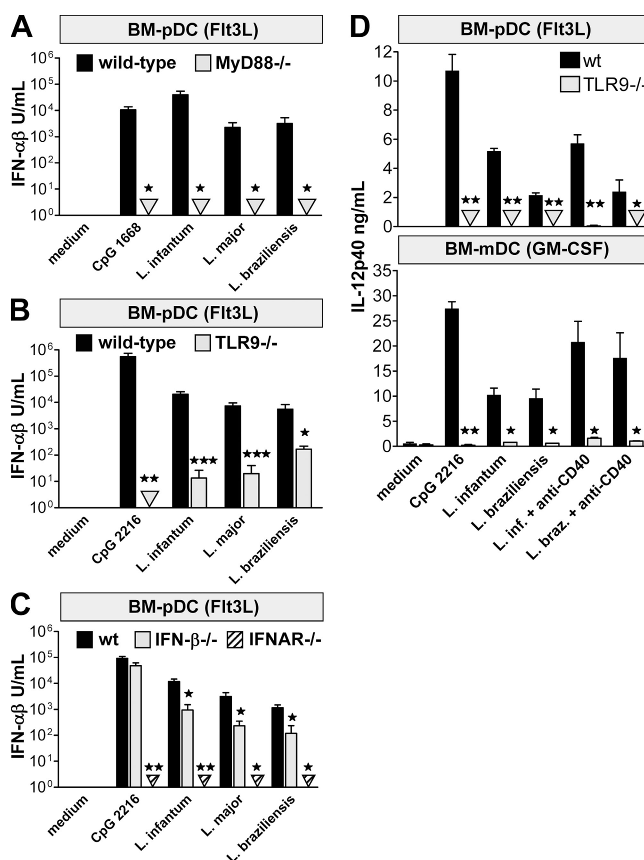

Figure 2. Receptors involved in *Leishmania*-induced expression of IFN-α/β and/or IL-12p40. Sorted BM-pDCs (from Flt3L-BM culture) and sorted immature BM-mDCs (from GM-CSF-BM culture) of C57BL/6 WT, MyD88–/–, TLR9–/–, IFN-β–/–, or IFNAR–/– mice were analyzed. After stimulation with 1 μM CpG ODN 2216, *L. infantum*, *L. major*, or *L. braziliensis* promastigotes (MOI = 3) for 48 h, the (A–C) IFN-α/β content (VSV bioassay) or the (D) IL-12p40 content (ELISA) of the respective culture supernatants was determined. Mean ± SEM of three (A and C), eight (B), or two independent experiments (D). ▼, not detectable. Significant differences between WT and KO cells are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

requires endogenous IFN-α/β signaling. No IFN-α/β was detectable in the cultures of sorted C57BL/6 BM-pDCs deficient for the IFNAR chain 1 ($IFNAR^{-/-}$) that were stimulated with *Leishmania* parasites or CpG ODN 2216. The IFN-α/β production triggered by *Leishmania* was also markedly reduced in $IFN\text{-}\beta^{-/-}$ pDCs (Fig. 2 C).

In both BM-pDCs and BM-mDCs, the IL-12p40 production elicited by the TLR9 ligand CpG ODN 2216 or by viable *Leishmania* promastigotes was entirely dependent on the presence of TLR9 (Fig. 2 D).

Collectively, these data illustrate that TLR9 is essential for the *Leishmania*-mediated induction of two major NK cell–activating cytokines (IFN-α/β and IL-12) in pDCs and mDCs. In addition, the generation of IFN-α/β by pDCs co-cultured with *Leishmania* promastigotes requires an IFNAR-dependent feedback loop that is partially maintained by IFN-β.

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), indicating that neither a specific parasite stage nor a productive infection of the host cells is required and that proteins are unlikely to be the only active component of *Leishmania* promastigotes. However, when pDCs were cultured with viable *L. infantum* promastigotes in the presence of DNase, we observed a significant (approximately fourfold) reduction of the IFN-α/β content in the culture supernatants (Fig. 3 A, top). This raised the possibility 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 ug/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*

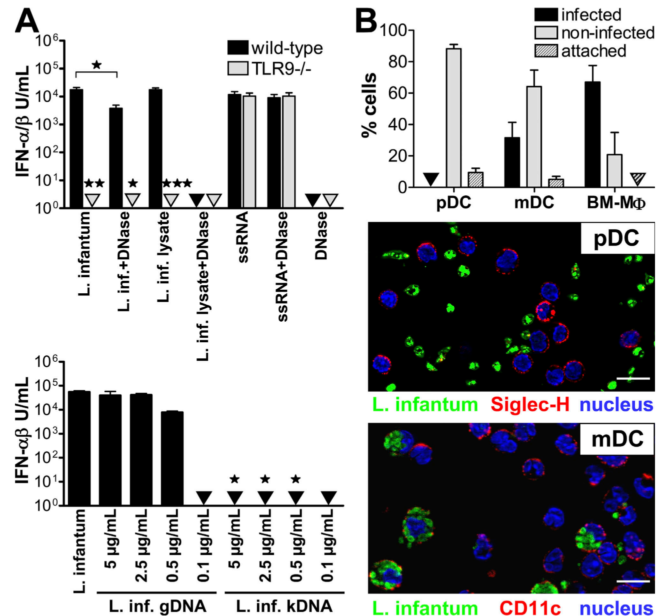

Figure 3. Parasite requirements for TLR9-dependent *Leishmania*-specific IFN-α/β production of pDCs. (A) IFN-α/β activity (VSV bioassay) in the culture supernatants of FACS-sorted C57BL/6 or $TLR9^{-/-}$ BM-pDCs stimulated for 48 h with viable *L. infantum* promastigotes (MOI = 3) in the absence or presence of 500 U/ml DNase I, *L. infantum* freeze-thaw lysate (MOI = 3, without or with DNase I treatment), 5 μg/ml of synthetic GU-rich ssRNA (without or with DNase I treatment), or 500 U/ml DNase I alone (A, top; mean ± SEM of three experiments) or with *L. infantum* promastigotes (MOI = 3), *L. infantum* gDNA, or *L. infantum* kDNA (A, bottom; mean ± SEM of two experiments). Significant differences (WT vs. KO, gDNA vs. kDNA) are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. (B) C57BL/6 BM-MΦ, sorted BM-mDCs, or sorted C57BL/6 BM-pDCs (from Flt3L-BM culture) were stimulated with *L. infantum* promastigotes (MOI = 3) for 16 h. After staining of the cell surface (anti-CD11c or anti–Siglec-H) and of the parasites, the number of infected cells, noninfected cells, or cells with attached parasites was determined microscopically by evaluation of at least 100 cells in different visual fields. Mean ± SEM of six (pDCs), four (mDCs), or three (BM-MΦ) separate experiments. Examples of the double immunofluorescence staining of pDCs or mDCs and *L. infantum* are shown in the lower panels. ▼, not detectable. Bar, 10 μM.

promastigotes might enter an acidic endosomal compartment of pDCs. However, multicolor fluorescent microscopy of sorted C57BL/6 pDCs (CD11b$^-$CD11c$^+$CD62L$^+$), mDCs (CD11b$^+$CD11c$^+$), 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$^{-/-}$ 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 our 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$^{-/-}$ 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$^+$ cells within the NK1.1$^+$CD3$^-$ splenic NK cell population was similar in WT and IFNAR$^{-/-}$ mice at the 12-h time point but was significantly reduced at 24 h of infection in the IFNAR$^{-/-}$ 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$^{-/-}$ 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–PDCA-1 mAb twice before infection, which led to an ∼80–90% reduction of the Siglec-H$^+$CD11c$^+$CD11b$^-$ 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*.

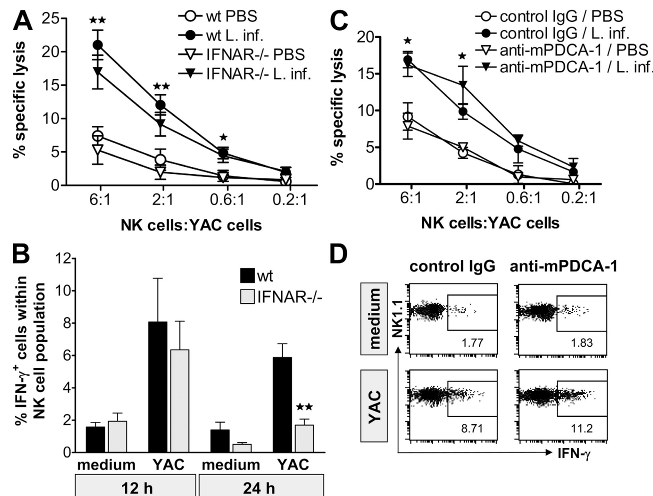

Figure 4. Role of IFN-α/β and pDCs for NK cell cytotoxicity and IFN-γ expression in the spleens of mice infected i.v. with 10^7 *L. infantum* promastigotes. (A and B) C57BL/6 WT versus IFNAR$^{-/-}$ mice. Mean ± SEM of four experiments with one to two mice per time point and mouse group. (C and D) Splenic pDCs of C57BL/6 mice were depleted by injection of 500 μg anti–mPDCA-1 mAb 24 and 4 h before infection with *L. infantum*. Control mice received rat IgG. Mean ± SEM of three experiments (C) or one of three experiments (D). (A and C) 24 h after injection of PBS or *L. infantum*, spleen cells were prepared and NK cell cytotoxic activity was measured. Infected WT mice were significantly different from PBS controls (*, $P < 0.05$; **, $P < 0.01$), but not from IFNAR$^{-/-}$ or anti–mPDCA-1–treated mice. (B and D) 12 and 24 h after infection, spleen cells were restimulated in medium ± YAC tumor cells (ratio 1:1) and stained for CD3$^-$NK1.1$^+$ NK cells and intracellular IFN-γ. **, $P < 0.01$ WT versus IFNAR$^{-/-}$.

The innate NK cell response to *L. infantum* requires IL-12 and CD11c^{high} 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/p40$^{-/-}$ (devoid of IL-12 and IL-23) or IL-12p35$^{-/-}$ 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^{high}MHCII^{+}CD11b^{+} splenic mDCs were ablated, whereas pDCs and all other CD11c^{int} cells remained unaffected (Fig. 6 A). In agreement with a previous report (52), we observed that the DT treatment not only depleted CD11c^{high}MHCII^{+} mDCs, but also MOMA-1^{+} metallophilic macrophages (MMs) and ERTR-9^{+} marginal zone macrophages (MZMs) in the spleen (Fig. 6 B). However, 5 d after DT treatment, CD11c^{high}MHCII^{+} mDCs had repopulated the spleen to a large, albeit varying extent in individually analyzed mice (Fig. 6 C), whereas ERTR-9^{+} MZMs remained completely depleted, and only very few MOMA-1^{+} MMs became visible in all 10 individually analyzed mice (Fig. 6 D). F4/80^{+} 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^{+} 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^{-}NK1.1^{+}) 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^{high} 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,

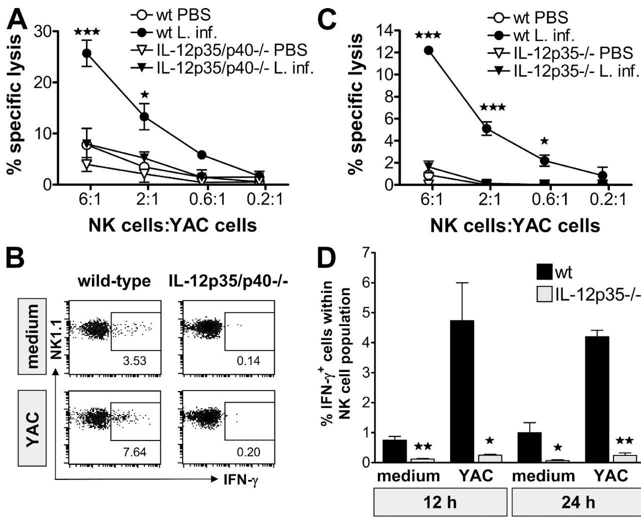

Figure 5. NK cell cytotoxicity and IFN-γ expression in the spleens of C57BL/6 WT versus IL-12p35/p40$^{-/-}$ and BALB/c WT versus IL-12p35$^{-/-}$ mice infected i.v. with 10^7 *L. infantum* promastigotes. (A and C) 24 h after injection of PBS or *L. infantum*, spleen cells were prepared and NK cell cytoxic activity was measured. Mean ± SEM of two experiments (two mice/group). (B and D) 12 and 24 h after infection, spleen cells of WT and KO mice were restimulated in medium ± YAC tumor cells (ratio 1:1) and stained for CD3^{-}NK1.1^{+} NK cells and intracellular IFN-γ. One (B) or mean ± SEM (D) of two experiments with one to two mice per time point and mouse group. (A, C, and D) The values obtained for infected WT mice are significantly different from WT PBS controls and from infected KO mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

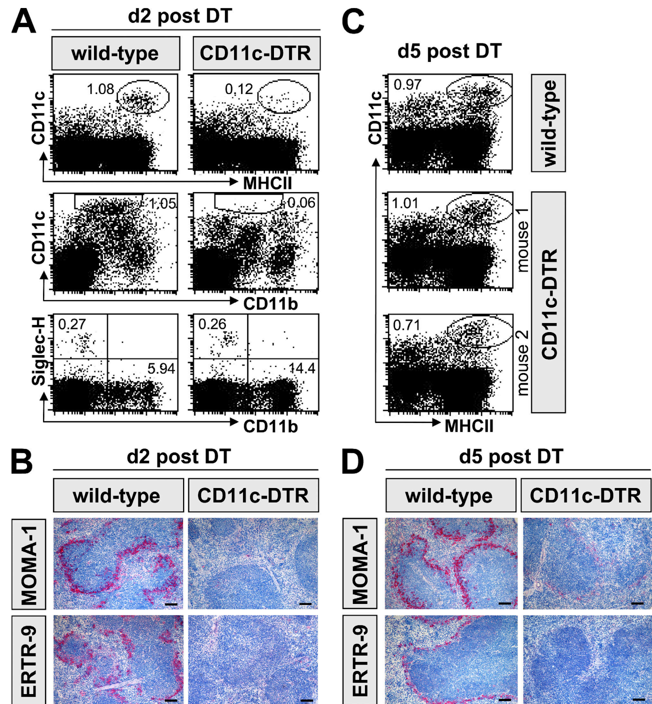

Figure 6. Phenotypic analysis of the spleens of C57BL/6 WT and CD11c-DTR/GFP transgenic mice 2 or 5 d after i.p. injection of DT and 12–24 h after i.v. infection with 10^7 *L. infantum* promastigotes. (A and C) Flow cytometric analysis of DC populations in the spleen. The percentage of the respective cell population is given in the plot panels. (B and D) Immunohistological staining of MZMs (ERTR-9^{+}) and MMs (MOMA-1^{+}) in the spleen. Nuclei were counterstained with Meyer's hemalaun. Bar, 100 μm. (A and B) One of five experiments, with two to three mice per mouse group. (C and D) One of two experiments, with 2–10 mice per mouse group. In C, two individual mice of the group of a total of 10 infected CD11c-DTR/GFP mice with different DC reconstitution are shown.

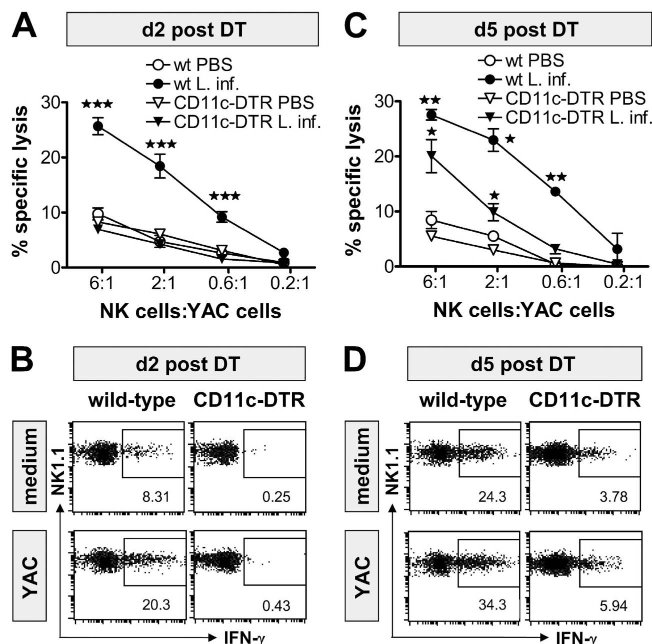

Figure 7. NK cell cytotoxicity and IFN-γ expression in C57BL/6 WT and CD11c-DTR/GFP transgenic mice 2 or 5 d after i.p. injection of DT and 12–24 h after i.v. infection with 10^7 *L. infantum* promastigotes or injection of PBS. (A) NK cell cytotoxicity of splenocytes at day 2 after DT. Mean ± SEM of five experiments, with two to three mice per time point and mouse group. ***, $P < 0.005$, WT *L. infantum* compared with WT PBS and CD11c-DTR *L. infantum*. (B) Intracellular IFN-γ staining of NK cells ($CD3^-NK1.1^+$) in splenocytes at day 2 after DT that were restimulated in medium ± YAC tumor cells (ratio 1:1). Staining of individual mice of one experiment is shown (day 5 analysis of the same experiment is illustrated in D). Similar results were obtained in five independent experiments. (C) NK cell cytotoxicity of splenocytes at day 5 after DT. Results of one of two similar experiments are shown, with 2–10 mice per mouse group. *, $P < 0.05$; **, $P < 0.01$, WT and CD11c-DTR *L. infantum*–infected mice compared with the respective PBS control. (D) Intracellular IFN-γ staining of NK cells in splenocytes at day 5 after DT (restimulated as in B). One of two independent experiments.

and NK cell cytotoxicity assays, which revealed that the magnitude of NK cell cytotoxicity solely correlated with the percentage of reconstitution of $CD11c^{high}MHCII^+$ mDCs ($r^2 = 0.77$; Fig. S6). MZMs and MMs were uniformly absent and the distribution and expression level of $CD11b^+$ or $F4/80^+$ 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 $CD11c^{high}MHCII^+$ mDCs ($r^2 = 0.66$; not depicted). We conclude that activation of NK cells during the innate phase of *L. infantum* infection requires IL-12 and the presence of $CD11c^{high}$ 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

The critical role of mDCs and IL-12 for the activation of NK cells in *L. infantum*–infected mice and the TLR9-dependent production of IL-12 by mDCs in vitro led us to investigate 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 $TLR9^{-/-}$ 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 $TLR9^{-/-}$ 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 $TLR9^{-/-}$ 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 $TLR9^{-/-}$ 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 $CD11c^{high}$ mDCs (~1% of all living spleen cells; see Fig. 6 A), but not in $F4/80^+$ macrophages (not depicted). As shown in Fig. 8 D, the *L. infantum*–induced production of IL-12p40 by $CD11c^{high}$ mDCs in the spleen and its accumulation in the plasma of WT mice were absent in $TLR9^{-/-}$ 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 $CD11c^{high}$ 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.

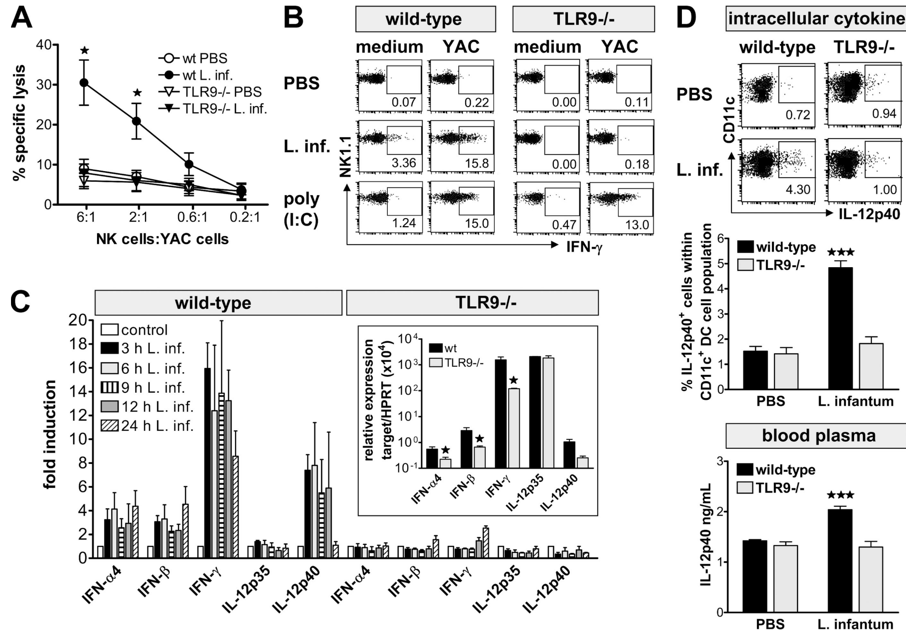

Figure 8. NK cell cytotoxicity, NK cell IFN-γ production, and cytokine expression in the spleens of WT and TLR9$^{-/-}$ mice infected i.v. with 10^7 *L. infantum* promastigotes. (A) NK cell cytoxicity of splenocytes at 24 h after infection. Mean ± SEM of four independent experiments. *, $P < 0.05$, WT *L. infantum* compared with WT PBS or TLR9$^{-/-}$ *L. infantum*. (B) 12 h after injection of *L. infantum*, PBS or poly (I:C) (50 μg, i.p.) spleen cells of WT and TLR9$^{-/-}$ mice were restimulated in medium ± YAC tumor cells (ratio 1:1) and stained for NK cells (CD3$^-$NK1.1$^+$) and intracellular IFN-γ. One of three similar experiments. (C) IFN-α4, IFN-β, IFN-γ, IL-12p35, and IL12p40 mRNA expression in the spleens 3, 6, 9, 12, and 24 h after infection. PBS control mice were set as 1 (mean ± SEM of three experiments; in each experiment two mice per time point and mouse group were analyzed by real-time RT-PCR with triplicate determinations for each gene). Inset: Relative cytokine mRNA expression levels (compared with the mHPRT-1 housekeeping gene) in the spleen 6 h after infection (mean ± SEM of three experiments). (D) Top panels: 12 h after injection of PBS or *L. infantum*, spleen cells of WT and TLR9$^{-/-}$ mice were restimulated in medium and stained for CD11c$^+$ DCs and intracellular IL-12p40 protein (one of three similar experiments; the percentage of IL-12p40$^+$ cells is indicated in the panels; mean ± SEM of three independent experiments is shown in the graph below). ***, $P < 0.001$, WT *L. infantum* compared with WT PBS and TLR9$^{-/-}$ *L. infantum*. Bottom: IL-12p40 plasma levels of 12-h-infected or PBS control mice as measured by ELISA (mean ± SEM of three independent experiments). ***, $P < 0.001$, WT *L. infantum* compared with WT PBS and TLR9$^{-/-}$ *L. infantum*.

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, CD11c^{high} 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 eukaroytic 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.

CD11c^{high} mDCs, IL-12, and TLR9-dependent NK cell activation

CD11c-DTR/GFP transgenic mice (51) are currently the only mouse model to eliminate CD11c^{high} DCs. To the best of our knowledge, this is the first study in which these mice have been used to demonstrate that CD11c^{high} 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 CD11c^{high} 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 CD11c^{high} DCs repopulated the spleen, although at these time points both MZMs and MMs were still absent. Thus, NK cell activation requires CD11c^{high} 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 CD11c^{high} 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 CD11c^{high}-depleted mice (Figs. 5, 7, and 8), and the lack of infection-induced IL-12p40 expression in CD11c^{high}-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 CD11c^{high} 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 CD11c^{high} 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$^+$) naive 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^{high} 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^{high} 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 Charles River Laboratories, and CD11c-DTR/GFP transgenic mice (15th generation backcross to C57BL/6; reference 51) were from The Jackson Laboratory. Breeding pairs of IL-12p35$^{-/-}$ mice (fifth generation backcross to BALB/c; reference 34) and IL-12p35/p40$^{-/-}$ mice (10th generation backcross to C57BL/6; reference 34) were provided by G. Alber (University of Leipzig, Leipzig, Germany) and H. Mossmann (Max Planck Institute for Immunobiology, Freiburg, Germany), respectively. IFNAR$^{-/-}$ (66), IFN-$\beta^{-/-}$ (67), MyD88$^{-/-}$ (68), and TLR9$^{-/-}$ mice (69) were backcrossed to C57BL/6 mice for 10, 13, 8, or 10 generations, respectively. All mice were housed under specific pathogen-free conditions and used at the age of 6–12 wk.

Promastigotes of *L. major* (MHOM/IL/81/FEBNI; reference 70), *L. infantum* (MHOM/00/98/LUB1; reference 71), and *L. braziliensis* (MHOM/BR/94/H-3227; reference 72) were grown from amastigotes isolated from skin lesions of BALB/c (*L. major* and *L. infantum*) or iNOS$^{-/-}$ (*L. braziliensis*) mice and propagated in vitro (70).

For infection, mice were injected i.v. in the retro-orbital vein or in the tail vein with 300 µl PBS or 10^7 stationary phase *L. infantum* promastigotes in 300 µl PBS. The animal experiments were approved by the animal welfare committee of the Regierungspräsidium Freiburg.

In vivo treatment

To deplete pDCs, mice were i.p. injected with 500 µg rat anti–mPDCA-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 infection, spleen cells were analyzed for the presence of CD11b$^-$CD11c^{int}Siglec-H$^+$ cells to control depletion. To deplete mDCs, CD11c-DTR/GFP mice received an i.p. injection of DT (4 ng/g body weight; Sigma-Aldrich) 1 or 4 d before *L. infantum* infection. The reduction of CD11c^{high}MHCII$^+$ mDCs was controlled by FACS analysis. The depletion of macrophages in DT-treated CD11c-DTR/GFP mice was monitored by immunohistology of the spleen. For activation of NK cells in vivo, mice received i.p. 50 µg poly(I:C) (Sigma-Aldrich).

FACS analysis

For surface phenotyping and cell sorting, the following unconjugated, fluorochrome (FITC-, PE-, or APC-) -labeled or 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–I-A/I-E (M5/114.15.2), anti-CD40 (3/23), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-NK1.1 (PK136), anti-CD49b (DX5), anti-CD3 (145-2C11), anti–mPDCA-1(Miltenyi Biotec), and anti–Siglec-H (440c; Hycult Biotechnology). Biotinylated antibodies were detected by streptavidin-APC or streptavidin-PE (BD Biosciences). The specificity of the stainings was verified by the use of isotype control mAbs. Propidium iodide was included at 1 µg/ml in the final wash to detect dead cells. All analyses were performed on a FACSCalibur (BD Biosciences) applying the CELLQuest Pro software. The FL3 channel was used to exclude propidium iodide$^+$ dead cells.

Generation and purification of pDCs and mDCs

Splenic pDCs and mDCs. After collagenase D (Roche Diagnostics) treatment, splenic DCs were enriched from total spleen cells by positive selection of CD11c$^+$ cells using anti-CD11c MicroBeads and AutoMACS (Miltenyi Biotec). pDCs were obtained by MoFlo sorting (Cytomation Inc.) of CD11b$^-$CD11c^{int}Gr-1$^+$ cells, and mDCs were obtained by sorting of CD11b$^+$CD11c^{high} cells (purity ≥95%). Purified mDCs also expressed MHC class II.

BM-pDCs. BM-pDCs were generated from total BM cells in the presence of Flt3L (73). After incubation in red blood cell lysis buffer (Sigma-Aldrich), BM cells were cultured in complete RPMI with 100 ng/ml rmFlt3L (R&D Systems) for 7–8 d at 2×10^6 cells/ml (25 cm^2 cell culture flasks, 5 ml). At day 4, 2.5 ml of medium per flask was replaced by 2.5 ml of fresh medium with 50 ng/ml Flt3L. After 7–8 d, 75–95% of the cells were CD11c$^+$. pDCs (CD11b$^-$) represented 57–77% and mDCs (CD11b$^+$) represented 23–43% of the CD11c$^+$ population (unpublished data). At day 7 or 8, CD11b$^-$CD11c$^+$CD62L$^+$ BM-pDCs were purified by MoFlo sorting (purity >95%). The purified pDCs were readily stained with anti-B220 and the pDC-specific mAbs anti–Siglec-H (74) and anti–mPDCA-1 (15 and unpublished data).

BM-mDCs. BM-mDCs were either sorted as CD11c$^+$CD11b$^+$ cells from Flt3L-expanded BM-cultures (see above) or generated from BM cells incubated with rmGM-CSF (75). GM-CSF–expanded BM cultures (day 8) contained ~80% CD11b$^+$CD11c^{high} mDCs, which were further purified as immature CD11b$^+$CD11c$^+$CD86^{low} mDCs by MoFlo sorting (purity ≥96%).

Stimulation of pDCs and mDCs

pDCs and mDCs were cultured in 96-well (10^5 cells/well, 250 µL), 24-well (10^6 cells/well, 1 ml), or 24-transwell tissue culture plates (10^6 cells/well; 0.4-µm pore size, 700 µL; Corning Costar) at 37°C and 5% CO_2/95% humidified air using RPMI 1640 (Invitrogen) that was supplemented with 50 µM 2-ME, 1% nonessential amino acids, 1 mM sodium pyruvate, 100 µg/ml kanamycin sulfate, and 10% FCS (PAA Laboratories) for pDC cultures or supplemented as described previously (29) plus 10% FCS (PAA) for mDC and all other cell cultures. Cells were activated for 12–72 h with 1 µM CpG ODN 2216 (Thermo Electron), 200 ng/ml LPS (*Escherichia coli* O111:B4; Sigma-Aldrich), 50 ng/ml poly(I:C) (Sigma-Aldrich), 20 ng/ml rmIFN-γ (provided by G. Adolf, Boehringer Ingelheim, Vienna, Austria), 5 µg/ml anti-mCD40 (clone 3/23; BD Biosciences), UV-inactivated HSV-1 (provided by T. Stamminger, University of Erlangen, Erlangen, Germany), *Leishmania spp.* promastigotes (logarithmic stage or stationary growth phase; parasite/pDC ratio [MOI] = 3:1, unless indicated differently), 160 mJ/cm^2 of UV-irradiated *L. major* or *L. infantum* promastigotes (MOI = 3:1), *Leishmania spp.* antigen (freeze-thaw lysates of promastigotes; MOI = 3:1), 0.1–5 µg/ml gDNA or kDNA of *L. infantum* promastigotes or with GU-rich ssRNA double-right complexed with LyoVec (5µg/ml; ssRNA-DR/LyoVec; InvivoGen). In some experiments, ssRNA, *Leishmania* DNA, or *Leishmania* lysates were digested with 500 U/ml of bovine pancreas DNase I (Sigma-Aldrich) before their addition to the pDC cultures according to the manufacturer's protocol. gDNA of *Leishmania spp.* was prepared by proteinase

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). kDNA 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, Institute Curie, Paris, France) was used as a standard or to ascertain that all antiviral activity in the supernatants was due to IFN-α/β.The content of IFN-α (including IFN-α1, IFN-α4, IFN-α5, IFN-α6, 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. NO_2^- 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-DX5$^+$), the cells were fixed with CytopermCytofix (BD Biosciences) for 20 min 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 (Cl: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 Lab. Glassware), whereas macrophages were directly stained in LabTek Permanox Chambers (Nalge Nunc Int.). For double immunofluorescence staining, the cells were blocked with PBS/1% BSA and incubated with biotinylated anti-CD11c (HL3; mDCs), anti–Siglec-H (pDCs), or anti-CD11b (macrophages) antibodies (BD Biosciences), followed by Cy3-conjugated streptavidin (Invitrogen). Internalized *Leishmania* parasites were stained after fixation in 3% paraformaldehyde and permeabilization in 1% saponin using human anti–*L. major* antiserum (78) and fluorescein–labeled anti–human IgG $(Fab')_2$ fragments (Dianova). Vectashield (containing DAPI to stain the nuclei; Vector Laboratories) -mounted slides were analyzed with an ApoTome-equipped Axioplan2 microscope connected to an AxioCam-MR digital camera (Carl Zeiss MicroImaging, Inc.).

Immunohistology

Immunohistochemistry of splenic acetone-fixed cryosections (5–6 μm) were performed using unconjugated rat anti-F4/80 (CI:A3-1), rat anti–mouse macrophage (MOMA-1), and rat anti–SIGN-R1 (ERTR-9) mAbs (all from BMA Biomedicals), followed by biotin-conjugated $(Fab')_2$ fragments of mouse anti–rat IgG or goat anti–rat IgM (μ chain specific; both from Dianova), alkaline phosphatase–conjugated streptavidin (DakoCytomation), and by a red alkaline phosphatase substrate (Vector Laboratories). Sections were counterstained with Meyer's hemalaun, mounted with Aquatex (Merck), and analyzed by light microscopy (Axioskop 2 plus; Carl Zeiss MicroImaging, Inc.).

RNA preparation and PCR

Total RNA was prepared using the RNeasy extraction kit (QIAGEN). Contaminant gDNA was removed with DNase (DNAfree; Ambion). The presence of gDNA was excluded by performing a PCR reaction with 1 μL of the RNA sample as template and primers for mouse β-actin (sense: 5′-CACCCGCCACCAGTTCGCCA-3′; antisense: 5′-CAGGTCCC-GGCCAGCCAGGT-3′). Total RNA (1–10 μg) was reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems). Subsequent real-time PCR was performed on an ABI Prism 7900 sequence detector (Applied Biosystems) using Taqman Universal Mastermix and Assays-on-Demand (Applied Biosystems), which include forward and reverse primers and the FAM-labeled probe for the target gene, respectively. The following assays were used: murine hypoxanthine guanine phosphoribosyl transferase 1 (mHPRT-1; Mm00446968_m1), mIFN-α2 (Mm00833961_s1), mIFN-α4 (Mm00833969_s1), mIFN-α5 (Mm00833976_s1), mIFN-α6 (Mm01258374_s1), mIFN-α9 (Mm00833983_s1), mIFN-α11 (Mm01257312_s1), mIFN-α12 (Mm00616656_s1), mIFN-α13 (Mm00781548_s1), mIFN-α14 (Mm01703465_s1), mIFN-β (Mm00439546_s1), mIL-12p35 (Mm00434165_m1), mIL-12p40 (Mm00434170_m1), mIFN-γ (Mm00801778_m1), and TLR9 (Mm00446193_m1). Each cDNA was amplified and measured in triplets with 50–100 ng cDNA per well in a reaction volume of 15 μL and 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 $= 2^{-(C_{T(target)} - C_{T(mHPRT-1)})}$.

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 10^7 *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. Fig. S5 illustrates the percentage of NK cells and the PMA/ionomycin-induced expression of IFN-γ protein by NK cells in the spleens of 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 996/3-1, 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.

Submitted: 19 June 2006
Accepted: 2 March 2007

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