ORIGINAL ARTICLE

Innate Lymphoid Cell 1– and NK Cell–Derived, Early IFNg Release Depends on ICER and Promotes Protection against *Leishmania major* Infection

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Innate lymphoid cells (ILCs) participate in different skin diseases. Cutaneous leishmaniasis evokes T helper 1/cytotoxic T cell 1–dominated immunity, whereas in immune-compromised individuals, a T helper 2/regulatory T cell/T helper 17 immune response dominates. Only a few prior studies investigated the role of ILC in leishmaniasis. We show that after physiologic low-dose infection with *Leishmania major*, both lesional NK cell and ILC1 numbers strongly increase. In addition, early lesional IFNγ production derives from type I ILCs. Genetic ablation of both NK cells and ILC1 (NK/ILC1$^{\Delta}$ mice) led to reduced early IFNγ expression, with increased pathology, higher parasite burdens, and delayed recovery. Furthermore, expression of ICER is important for disease outcome because *Icer*$^{-/-}$ mice exhibited significantly larger lesions. Interestingly, mice that lack ICER specifically in NK cells and ILC1 phenocopied the worsened disease outcome of *Icer*$^{-/-}$ mice, whereas ICER deficiency in T cells or macrophages alone failed to do so. In line, ICER deficiency in NK cells/ILC1 resulted in higher lesional parasite burden with fewer IFNγ-positive ILC1 than in control mice. Thus, our data show that both NK cells and ILC1 contribute to early parasite control by releasing IFNγ. ICER expression by ILC1 promotes recruitment of IFNγ^{+} ILC1 in *Leishmania* infections important for development of protection against this important pathogen.

Keywords: ICER, ILC, *Leishmania major*, NK cell, IFNγ

INTRODUCTION

Cutaneous leishmaniasis is characterized by localized, papulous, or ulcerative skin lesions in a variety of mammals, including humans and mice. The disease is evoked by the parasite *Leishmania major*, transmitted by sand fly bites. The parasite's main host cell is the dermal macrophage (denoted as MΦ). After their inoculation, parasites are quickly internalized by neutrophils and MΦ, in which they transform into the amastigote life form to then reside in parasitophorous vacuoles. Later, after activation of dendritic cells (DC) due to their infection, development of a T cell–medicated immunity provides protection. In healthy individuals, parasite killing is initiated by IFNγ-mediated activation of MΦ to produce nitric oxide (Sacks and Noben-Trauth, 2002), whereas progressive disease is associated with T helper (Th)2/regulatory T cell/Th17–dependent immune responses. As such, priming of protective IFNγ-producing CD4^{+} Th1 cells and CD8^{+} cytotoxic Tc1 cells by DC is dependent on IL-12 and IL-1α/β (Filippi et al, 2003; Sacks and Noben-Trauth, 2002; von Stebut et al, 2003).

NK cells are part of the innate immune system capable of killing infected cells and of producing various cytokines, such as IFNγ. The role of NK cells in cutaneous leishmaniasis is still controversial (Cavalcante et al, 2022). Whereas some studies in patients provided evidence for an association with cure, others showed disease progression in relation to a strong inflammatory response. A divergent role was also seen depending on the type of disease: diffuse leishmaniasis with higher parasite burden was associated with low NK cell numbers, whereas localized forms harbored higher numbers (Cañeda-Guzmán et al, 2014). In mice, NK activation was achieved by toll-like receptor 9–mediated IL-12 release from *L major*–stimulated DCs (Liese et al, 2007). It was thought that NK-derived IFNγ but not their cytotoxic activity activates MΦ to eliminate the parasite (Liese et al, 2007). In vivo models genetically devoid of NK cells have not been available to finally solve this question.

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Abbreviations: DC, dendritic cell; ILC, innate lymphoid cell; Th, T helper

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Innate lymphoid cells (ILCs) are found in various tissues, including the skin. ILC share features with Th cells but do not express the T cell antigen receptor (Vivier et al, 2018). In functional and developmental analogy to Th1, Th2, and Th17 subsets, ILC are subdivided into 3 groups: type 1 ILC (comprise NK cells and ILC1), type 2 ILC (ILC2), and type 3 ILC (ILC3 and lymphoid tissue inducer cells) (Eberl et al, 2015). Under steady-state conditions, skin mainly harbors ILC2, fewer numbers of NK cells/ILC1, and very low numbers of ILC3 (Kim et al, 2016). ILC2 cells have been associated with the pathophysiology of atopic dermatitis, whereas ILC3 are important players in disease manifestation of psoriasis through IL-17 (Kim et al, 2016). The contribution of ILC1 to skin immunity is less clear. ILC1 express Tbet and IFNγ; recent evidence suggests involvement in allergic contact dermatitis (Kim, 2015). In cutaneous leishmaniasis, the role for ILC1 cells, which can produce Tbet and IFNγ in an antigen-independent manner, has not been addressed so far.

Even though type I ILC respond to intracellular pathogens, contribute to tumor and virus control, and share the expression of IFNγ, their role, especially that of ILC1, in the context of *L major* infection is still unclear. In this study, we assessed the role of NK cells/ILC1 in cutaneous leishmaniasis by utilizing mice devoid of NKp46^{+} cells. In our hands, absence of NK cells/ILC1 in skin led to strongly enhanced pathology after physiologic low-dose infection with *L major*, associated with higher parasite burdens and a lack of early IFNγ production at lesional sites. In addition, as a mechanism through which type I ILC are activated, we show that ICER expression in ILC1 (and NK cells)—by cell-specific deletion of ICER on type I ILCs—promoted protection against this important pathogen.

RESULTS

Increase of IFNγ-producing type I ILC in skin after *L major* infection

To investigate whether ILC1 and NK cells are involved in the response to *Leishmania* infection, we investigated the frequency of these cells after physiologic low-dose, intradermal infection with 10^3 infectious-stage parasites. To this aim, wild-type C57BL/6 mice were infected intradermally, and the frequency of NK cells and ILC1 was assessed over time. We found that the numbers of lineage-negative, CD11b^{neg}, Rorγt^{neg}, NKp46^{+} cells—that include NK cells (Eomes^{hi}) and ILC1 (Eomes^{low}) cells—were absent from ear skin under steady-state conditions, as previously described (Torcellan et al, 2024). However, during the course of infection, these numbers increased significantly and peaked at week 9 at around the time when skin lesions clearly start to resolve (Figure 1a).

To next assess the contribution of NK cells and ILC1 to local IFNγ production in lesion sites (and thus parasite control), we isolated dermal cells at different time points after low-dose infection. Surprisingly, we found a dichotomic expression of IFNγ: the number of IFNγ^{+} cells strongly increased at week 3 (at a time when lesions are typically not visible yet) and again in week 12 during lesion resolution (Figure 1b). Subsequently, IFNγ^{+} cells were further separated by excluding cells staining for CD3. Interestingly, in week 3, we found that a large fraction of IFNγ-producing cells were

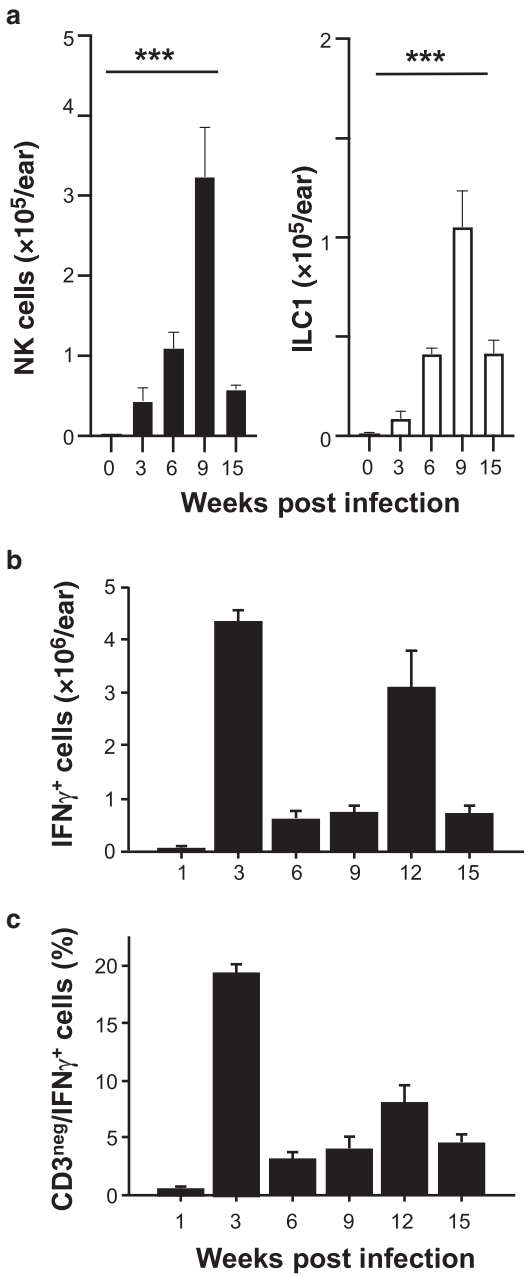

Figure 1. In *Leishmania major* infections, lesional numbers of NK cells and ILC1 increase, and early IFNγ is primarily produced by CD3-negative cells. Wild-type C57BL/6 mice were infected with physiologic low-dose inocula of *L major* (10^3 metacyclic promastigotes/ear). (**a**) At indicated time points, lesions were harvested, and NK cells as well as ILC1 were enumerated through flow cytometry. NK cells were identified as viable CD45^{pos} lineage (TCRβ, TCRγδ, CD3, CD5, CD19, FcεR1)-negative NKp46^{pos} CD11b^{neg} Rorγt^{neg} Eomes^{pos} cells, and ILC1 were gated as viable CD45^{pos} Lin^{neg} NKp46^{pos} CD11b^{neg} Rorγt^{neg} Eomes^{neg} cells. (**b**) After low-dose infection of C57BL/6 mice, lesions were harvested, and cell infiltrates of lesional skin were assessed for IFNγ-producing CD45^{+} cells. (**c**) The frequency of CD3-negative cells among all IFNγ^{+} cells was determined. All data are shown as mean ± SEM, with n = 5 mice per group. Statistical differences between groups were calculated using 2-way ANOVA (***$P \leq .001$). ILC, innate lymphoid cell.

CD3^{neg} cells, whereas at later time points, IFNγ was predominantly produced by T cells (Figure 1c). These findings suggest that early IFNγ in cutaneous leishmaniasis is derived

from cells of the innate immune system, most likely type I ILC, which both are capable of producing IFNγ after *L major* infection independent of prior antigen priming.

Worsened disease outcome in the absence of NKp46^{+} cells

We next assessed the contribution of ILC1 and NK cells to protection against physiologic low-dose infection with *L major*. To this aim, we utilized NKp46-Cre x DTA mice lacking NK cells and ILC1 (NK/ILC1$^{\Delta}$), due to Cre-dependent expression of diphtheria toxin. NK/ILC1$^{\Delta}$ mice were infected intradermally with physiologic low-dose inocula of 10^{3} metacyclic *L major* parasites, and disease outcome was monitored for several weeks. Interestingly, over the course of several weeks, NK/ILC1$^{\Delta}$ mice developed significantly larger lesions and showed delayed healing compared with NKp46-Cre control mice (Figure 2a). In line, parasite numbers in lesions at weeks 6 and 9 were significantly increased (Figure 2b); the difference in lesional parasite numbers was >10-fold. Parasite dissemination into visceral organs such as spleen was not dramatically altered, but again, in these organs, NK/ILC1$^{\Delta}$ mice harbored more parasites (Figure 2b).

To confirm that NK/ILC1$^{\Delta}$ skin contains fewer type 1 ILCs, NK cell and ILC numbers were assessed under steady-state conditions at various time points after infection. To this aim, inflammatory immune cells were isolated from infected lesional skin. The numbers of NK cells and ILC1 were indeed dramatically reduced in NK/ILC1$^{\Delta}$ mice at all times (Figure 2c), whereas the ILC2 frequency was unaffected.

We next investigated the frequency of IFNγ^{+} cells of all viable CD45^{+} cells in skin lesions of NK/ILC1$^{\Delta}$ mice and control C57BL/6 mice starting as early as week 3, a time point at which lesions are barely visible. We found that the numbers of IFNγ-producing CD45^{+} cells increased over time (Figure 3a). Three weeks after infection, the numbers of IFNγ-producing cells was significantly lower in skin lesions of NK/ILC1$^{\Delta}$ mice than in those of control mice. Week 6 after infection, numbers were similar, but in week 9, the numbers were significantly higher in the mice lacking NK and ILC1 than in control mice (Figure 3a).

We also calculated the number of NK cells, ILC1, and CD3^{+} T cells staining positive for IFNγ in lesions over time (Figure 3b). As described, the numbers of IFNγ^{+} NK cells increased early after infection (Liese et al, 2007). It appeared that the reduced number of total IFNγ^{+} cells as shown in Figure 3a was due to a lack in both NK cells and ILC1 in NK/ILC1$^{\Delta}$ mice, whereas higher numbers of IFNγ-producing T cells were found at later time points, indicating a compensatory mechanism with enhanced T cell reactivity.

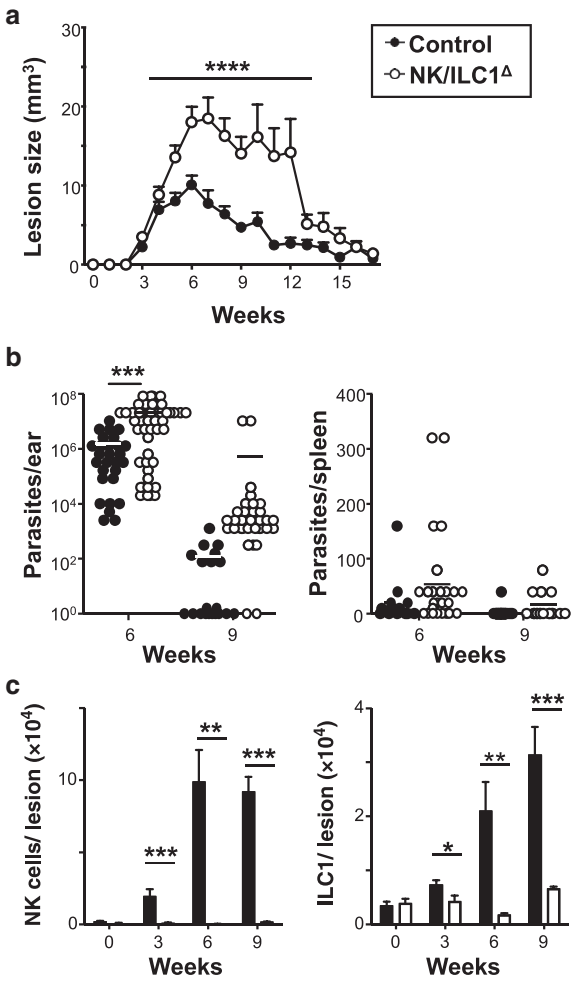

Figure 2. Worsened disease outcome in the absence of NK cells and ILC1 after infection with *L major*. NKp46-Cre × DTA (NK/ILC1$^{\Delta}$) mice and controls were infected intradermally with low-dose *L major* inocula of 10^{3} promastigote parasites/ear. (**a**) Ear lesion sizes were assessed in 3 dimensions and calculated as ellipsoid (mean ± SEM, pooled data from n = 5 mice per group, n = 4 independent experiments, statistical significance was assessed using 2-way ANOVA; $^{****}P \leq .0001$). (**b**) Parasite burdens in ear skin and spleen were determined by limiting dilution assay. Data are shown as individual measurements, and bars indicate means (pooled data of n = 5–10 mice per time point, n = 2 independent experiments, statistical significance was assessed using the unpaired *t*-test; $^{***}P \leq .001$. (**c**) NK cell and ILC1 numbers in lesional skin of infected mice were determined by FACS. Viable CD45^{pos} Lin^{neg} NKp46^{pos} CD11b^{neg} Rorγt^{neg} Eomes^{pos} cells were marked as NK cells, and CD45^{pos} Lin^{neg} NKp46^{pos} ILC1 were identified as CD11b^{neg} Rorγt^{neg} Eomes^{neg} cells (mean ± SEM, pooled data with n = 1–6 mice per group and n = 1–3 independent experiments; $^{*}P \leq .05$, $^{**}P \leq .01$, and $^{***}P \leq .001$, unpaired *t*-test). ILC, innate lymphoid cell.

The transcription factor Hobit does not control ILC1 differentiation in the skin

Next, in an attempt to distinguish between NK cell activity and that of ILC1, we utilized mice lacking Hobit (Hobit^{ko}), a transcription factor associated with ILC1 differentiation and maturation (Friedrich et al, 2021). After low-dose infection with *L major*, Hobit^{ko} mice did not show an altered disease outcome and parasite burdens comparable with those of control mice (Supplementary Figure S1a and b). Despite reported lower numbers of ILC1 in the liver (Friedrich et al, 2021), lesional skin of infected Hobit^{ko} mice exhibited normal numbers of these cells during the first weeks of infection. In contrast, 9 weeks after infection, both NK cells and ILC1 were significantly reduced but were not deleted (Supplementary Figure S1c). We have previously observed a Hobit-independent differentiation of skin-resident NK cells and ILC1 during viral and bacterial infection (Torcellan et al, 2024). In this study, we did not test whether Hobit was expressed by NK/ILC1 or whether it was required in a cell-intrinsic manner. Of note, lack of *Hobit* did not alter the effector function of ILC1 or NK cells as determined by the frequency of IFNγ^{+} NK cells or ILC1 (data not shown).

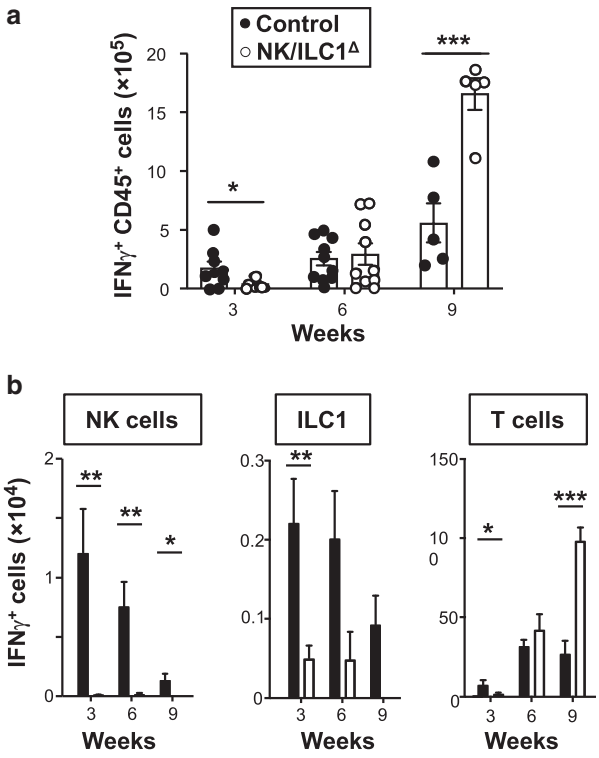

Figure 3. Early production of IFNγ by NK cells and ILC1 cells contributes to parasite clearance. Lesional skin of NK/ILC1$^{\Delta}$ mice or controls was harvested at indicated time points after infection with 10^3 *L major* parasites. (**a**) Absolute numbers of IFNγ-expressing CD45$^+$ cells at indicated time points after low-dose *L major* infection are shown. (**b**) CD45$^+$ cells were further separated into numbers of lineage (TCRβ, TCRγδ, CD3, CD5, CD19, FcεR1)-negative IFNγ$^+$ NK cells and ILC1 or lineage$^+$ T cells. Data are shown as mean ± SEM, pooled from n ≥ 3 mice per time point, n = 1−2 independent experiments. Significance was assessed using the unpaired *t*-test (*$P \leq .05$, **$P \leq .01$, and ***$P \leq .001$). ILC, innate lymphoid cell.

Expression of ICER in NKp46$^+$ cells represses lesion development after parasitic infection

ICER belongs to a group of proteins that act as transcription repressors and binds to cAMP responsive elements such as CREM and CREB (Bohn et al, 2018; Raker et al, 2016). ICER thus attenuates cellular responses in various physiologic processes; we have recently shown that expression of ICER in melanoma-associated MΦ led to their polarization toward a noninflammatory M2 phenotype and promoted tumor growth (Bohn et al, 2018). When studying the role of ICER for disease outcome in cutaneous leishmaniasis, we observed that lesions of ICER-deficient mice were significantly, almost 2-fold larger, and lesion resolution was delayed compared with that of wild-type mice (Figure 4a).

Because ICER can be produced by a variety of immune cells, we next assessed cell-specific expression of ICER. Interestingly, mice with ICER deletion in T cells (CD4-Cre) or myeloid cells (LyM-Cre) did not exhibit changes in disease course compared with control mice (Figure 4b). In contrast, mice that lack ICER in NK cells and ILC1 utilizing NKp46-Cre mice phenocopied the worsened disease outcome as mice lacking ICER in the whole body (Figure 4c). Lesions of mice with NK cells/ILC1 unable to express ICER showed significantly increased pathology, especially early on after infection. In line, lesional parasite burdens displayed significantly higher number of parasites at the site of skin infection in week 6, whereas visceralization was not as strongly enhanced (Figure 4d).

Finally, to understand the reason for the lack of early disease control in mice with no ICER expression in type I ILC, we assessed lesional numbers of IFNγ-producing cells in mice lacking ICER on NK cells and ILC1 (Figure 5). Lesions harbored a significantly lower frequency of IFNγ-positive ILC1 (and not NK or CD4$^+$ T cells) than Cre-negative controls. This is in line with our data obtained with mice lacking NKp46$^+$ cells entirely, which also exhibited increased pathology and harbored reduced IFNγ$^+$ NK/ILC1 numbers in lesions associated with delayed recovery. Thus, ICER expression on ILC1 (and possibly NK cells) is essential for their early contribution to parasite control through IFNγ.

DISCUSSION

In cutaneous leishmaniasis, parasite control and healing require IFNγ production, which leads to MΦ activation and parasite elimination through mitric oxide (Sacks and Noben-Trauth, 2002). Our data provide proof that IFNγ production by ILC1 and NK cells in cutaneous leishmaniasis promotes protection in vivo. Without NK cells and ILC1, early ICER-associated, IFNγ-mediated control of parasite expansion was absent, resulting in more severe lesion development.

In line to previous findings, we found that the numbers of lineage-negative, CD11b^{neg}, Rorγt^{neg}, NKp46-positive cells—which include NK cells (Eomes^{hi}) and ILC1 (Eomes^{low}) cells—in the skin under steady-state conditions was absent (Kim et al, 2016; Torcellan et al, 2024); however, also as reported before, the frequency of IFNγ-producing NK cells increased at early time points after infection (Laabs et al, 2009; Scharton and Scott, 1993). With the tools available then, these authors did not distinguish between NK cells and ILC1; in our study, in week 9, when both cells peaked in numbers, one quarter of all lesional type I ILC were ILC1, whereas about three quarter appeared to be NK cells.

The role of NK cells in leishmaniasis is still somewhat unclear; mice devoid of NK cells were not available. Although it is known that an IL-12/toll-like receptor 9−dependent activation of NK cells through DC leads to IFNγ production (Liese et al, 2007), and parasite antigens clearly bind to NK cells (Prajeeth et al, 2011), antibody-mediated depletion of NK cells early on showed a strong effect on disease control by reducing IFNγ (Bogdan, 2012). It appeared that the cytotoxic activity of NK cells was less important. In addition, deletion of NK cells by anti-asialo-GM1 or anti-NK1.1 antibodies given within the first weeks after infection led to disease exacerbation (Laskay et al, 1993). However, on the other hand, it was also known that NK cells are capable of promoting inflammation in the context of infection. In addition, other data using mice with an absence of NK cells and T cells and readministration of T cells did not reveal similar results (Satoskar et al, 1999). These mice lacking NK cells (NK-T+) developed an efficient Th1-like response, produced significant amounts of IL-12 and IFNγ, and controlled cutaneous *L major* infection. By crossing NKp46-Cre mice with Rosa-DTA mice, we successfully generated mice genetically devoid of NK cells and

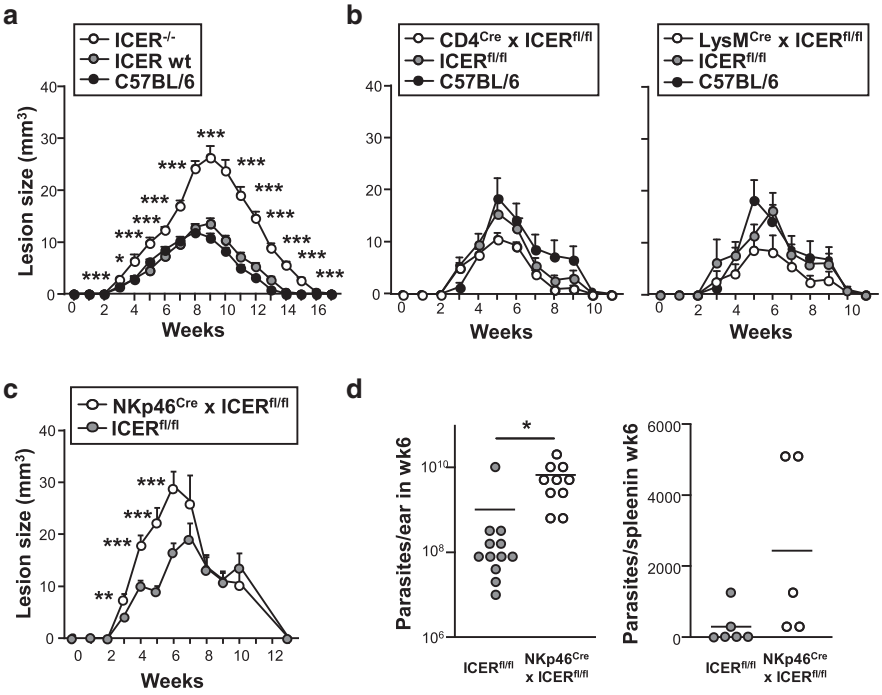

Figure 4. Mice with NKp46-specific but not MΦ or T cell–specific deletion of ICER expression—similar to ICER-deficient mice—exhibit worsened disease outcome in *L major* infection in vivo. *Icer*$^{-/-}$ mice or ICER$^{fl/fl}$ mice with selective deletion of ICER in all T cells (using CD4-Cre), in MΦ (using LysM-Cre), or in type I ILC (using NKp46-Cre) and appropriate controls (all on C57BL/6 background) were used. Groups of 5 mice were infected intradermally with physiologic 10^3 metacyclic promastigotes of *L major*. (**a**–**c**) Lesion development was assessed weekly using a caliper; lesion volumes are expressed as ellipsoid (mean ± SEM, pooled data of n = 1–2 [for **a** and **b**] and 4 independent experiments [for **c**] are shown; $^{**}P \leq .01$ and $^{***}P \leq .001$). (**d**) In wk 6, parasite burdens of mice with NKp46-specific deletion of ICER and respective controls were determined using limiting dilution assays. Data are shown as individual measurements, and bars indicate means (n ≥ 5 mice per time point, statistical significance was assessed using the unpaired *t*-test, $^{*}P \leq .05$). ILC, innate lymphoid cell; wk, week.

ILC1. In this setting, *L major* infection was uncontrolled and led to dramatically increased pathology and delayed healing, including higher parasite burdens. In line with previous findings by Liese et al (2007), we detected decreased IFNγ release, especially at an early time point (3 weeks), suggesting that both of these innate type I ILCs contribute to early parasite control. Interestingly, the resulting Th1 response at later stages after infection was significantly stronger, possibly indicating a compensatory mechanism.

The transcription factor Hobit has been proposed as a master transcription factor for tissue-resident memory T cells and ILC1s. Hobit^{ko} mice were shown to harbor lower numbers of ILC1 in the liver and to lack cytotoxic differentiation of ILC1 in other organs (Friedrich et al, 2021; Yomogida et al, 2021). Our data confirm that skin ILC1 induced by infection appear to be independent of the transcription factor Hobit (Torcellan et al, 2024). Thus, additional experimental models that allow for a separation of the roles of NK cells from those of ILC1 are needed to further study their respective contribution to anti-parasite immunity.

Even though more data are available in human cutaneous leishmaniasis on the functional importance of NK cells (including a direct activation or inhibition of NK cells by promastigotes, a suppression of NK cell numbers and activity during chronic, nonhealing infections, and a recovery of NK cell activity after treatment) (Bogdan, 2012), there is also some evidence for a role of ILC1 in protection against this important pathogen from humans. In patients, ILC1 (and ILC3) numbers in peripheral blood correlated with disease control, whereas, for example, in diffuse leishmaniasis, ILC2 numbers were associated with a tolerogenic state of these patients (Rodríguez et al, 2021).

In parallel to type I ILC, studies on the role of NK T cells in leishmaniasis are rare (Cavalcante et al, 2022). Compared with these, the much less frequent NK T cells share characteristics with both NK cells and T cells. They express the invariant TCR and CD1d and can produce a variety of cytokines. We have previously shown that after infection with *L major*, comparable numbers of skin-immigrating NK T cells in both susceptible BALB/c mice and resistant C57BL/6 mice were noted (Griewank et al, 2014). NK T cell–deficient C57BL/6 mice were better able to contain infection and showed decreased IL-4 production. In addition, low doses of the NK T cell–stimulating αGalCer analog applied at the time of infection led to disease exacerbation in wild-type C57BL/6 mice in an IL-4–dependent fashion but not in NK T-cell–deficient mice. Thus, it seems that in contrast to type I ILC, NK T cells have a different role for disease outcome.

cAMP is established as a universal regulator of metabolism (Raker et al, 2016). It controls both innate and adaptive immunity, including the function of monocytes/MΦ, neutrophils, DC, T and B cells, and NK cells. In NK cells, cAMP repression alters target cell lysis. Even though adenosine monophosphate is a central player in the signaling networks of many cells/diseases, we now describe that in cutaneous leishmaniasis, ICER expression appears to be important in NKp46^{+} cells, but not in MΦ or T cells. ICER-mediated activation of type I ILC was required for early parasite

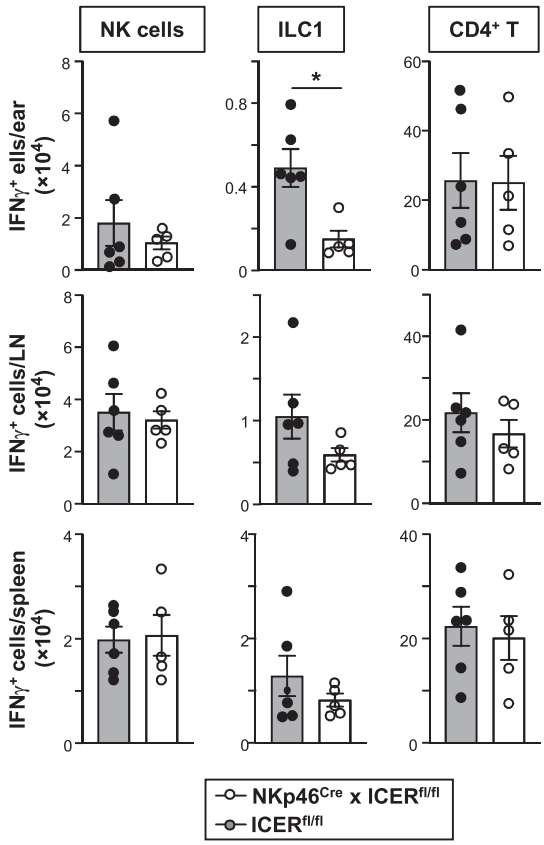

Figure 5. Decreased production of IFNγ by ILC1 in the absence of ICER on NKp46^{+} cells. Mice with no expression of ICER on NKp46^{+} cells (NK cells/ILC1) were infected with 10^3 *L major*. In week 6, lesional IFNγ^{+} NK cells, ILC1, and CD4^{+} T cells were enumerated using flow cytometry as described. Data are shown as mean ± SEM, with n = 5 mice per group. Statistical differences between groups were calculated using unpaired *t*-test (*$P \leq .05$). ILC, innate lymphoid cell.

control and clearly controlled lesion progression, associated with IFNγ release from ILC1.

In summary, we have shown that mice genetically devoid of NK cells and ILC1 exhibited increased pathology and reduced IFNγ expression associated with delayed recovery after physiologic low-dose infection. In wild-type mice, the numbers of both IFNγ-producing NK cells and ILC1 strongly increased early after infection, which appeared to be important for immediate parasite control. In addition, our data show that ICER expression on ILC1 (and less relevant on NK cells) promotes the recruitment of IFNγ^{+} ILC1 to the skin in *Leishmania* infections, which is important for the development of protective immunity against this important pathogen.

MATERIALS AND METHODS

Animals

C57BL/6 mice aged 6–8 weeks were purchased from Janvier. Mice devoid of type I ILCs were generated by crossing NKp46-Cre with Rosa-DTA mice (on C57BL/6 background). *Icer*$^{-/-}$ and *Icer*$^{fl/fl}$ mice were described previously (Kojima et al, 2008); cell-specific deletion of ICER was achieved by crossing *Icer*$^{fl/fl}$ mice with LysM-Cre, CD4-Cre, or Nkp46-Cre mice (Narni-Mancinelli et al, 2011). Hobit^{ko} mice were used as published previously (Friedrich et al, 2021).

Mice were housed under specific pathogen-free conditions in the Translational Animal Research Center of the Johannes Gutenberg-University (Mainz, Germany) and the Animal Husbandry Network of the Medical Faculty (Cologne, Germany). Animal housing and all the experimental procedures were authorized by the Animal Care and Use Committees of the Regions Rhineland Palatinate (23 170-07-G16-1-090) and the North Rhine–Westphalian State Agency for Nature, Environment, and Consumer Protection (Lanuvio, Germany) (81-02.04.2018.A209). All mouse lines used did not suffer from their gene modifications.

Parasites and infections

Amastigotes or metacyclic promastigotes of *L major* clone VI (MHOM/IL/Friedlin) were prepared as previously described (von Stebut et al, 2003). Amastigotes were isolated from infected ears of BALB/c mice. Groups of 5 mice were infected with physiologic low dose of 10^3 metacyclic *L major* promastigotes in a volume of 10 µl by intradermal injection into both ears using 0.3-mm-diameter needles. Lesion volumes were measured weekly in 3 dimensions and are reported as ellipsoids ([a/2 × b/2 × c/2] × 4/3 x π). Parasites present in lesional tissue were enumerated using a limiting dilution assay as previously described (von Stebut et al, 2003).

For measurement of antigen-specific cytokine production in infected mice, draining lymph node cells of infected mice were recovered, and single-cell suspensions were prepared. One million lymph node cells per 200 µl complete RPMI 1640 (Biochrome) were added to 96-well plates in the presence of 25 µg/ml soluble *L* antigen. Supernatants were harvested 48 hours after stimulation and assayed using ELISAs specific for IFNγ (R&D Systems) as well as IL-4 and IL-10 (BD Biosciences).

Flow cytometry

Inflammatory or steady-state myeloid cells in lesions were isolated by enzymatic digestion followed by mechanical disruption (Woelbing et al, 2006). Next, T cell, NK cell, and ILC1 numbers in lesional skin of infected mice were determined by FACS. ILCs were identified as viable CD45^{pos} lineage (TCRβ, TCRγδ, CD3, CD5, CD19, FcεR1)^{neg} cells (Friedrich et al, 2021; Vivier et al, 2018). Among these, NK cells were characterized as NKp46^{pos} CD11b^{neg} Rorγt^{neg} Eomes^{pos} cells, and ILC1s were gated as NKp46^{pos} CD11b^{neg} Rorγt^{neg} Eomes^{neg} cells. Intracellular staining for IFNγ was performed after fixation and permeabilization of cells. Cellular infiltrates were asssessd using an Attune flow cytometer. Relative and absolute numbers of infiltrating cells were calculated per ear.

Statistics

Statistical analysis was performed using StatView software and unpaired Students *t*-test.

ETHICS STATEMENT

All experiments with mice were done according to the guidance of the state of Rhineland Pfalz. Animal housing and all the experimental procedures were authorized by the Animal Care and Use Committees of the Regions Rhineland Palatinate (23 170-07-G16-1-090) and the North Rhine–Westphalian State Agency for Nature, Environment, and Consumer Protection (Lanuvio, Germany) (81-02.04.2018.A209).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author (esther.von-stebut@uk-koeln.de) upon request.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: EvS, AW, EV, GG; Data Curation: DL, XL, MR, SK-W; Investigation: DL, XL, MR, SK-W; Resources: TB, EV, GG; Funding Acquisition: AW, EvS; Supervision: AW, EvS; Writing − Original Draft Preparation: EvS, AW; Writing − Review and Editing: DL, XL, MR, SK-W, TBop, Tboh, EV, GG, AW, EvS

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2025.04.027.

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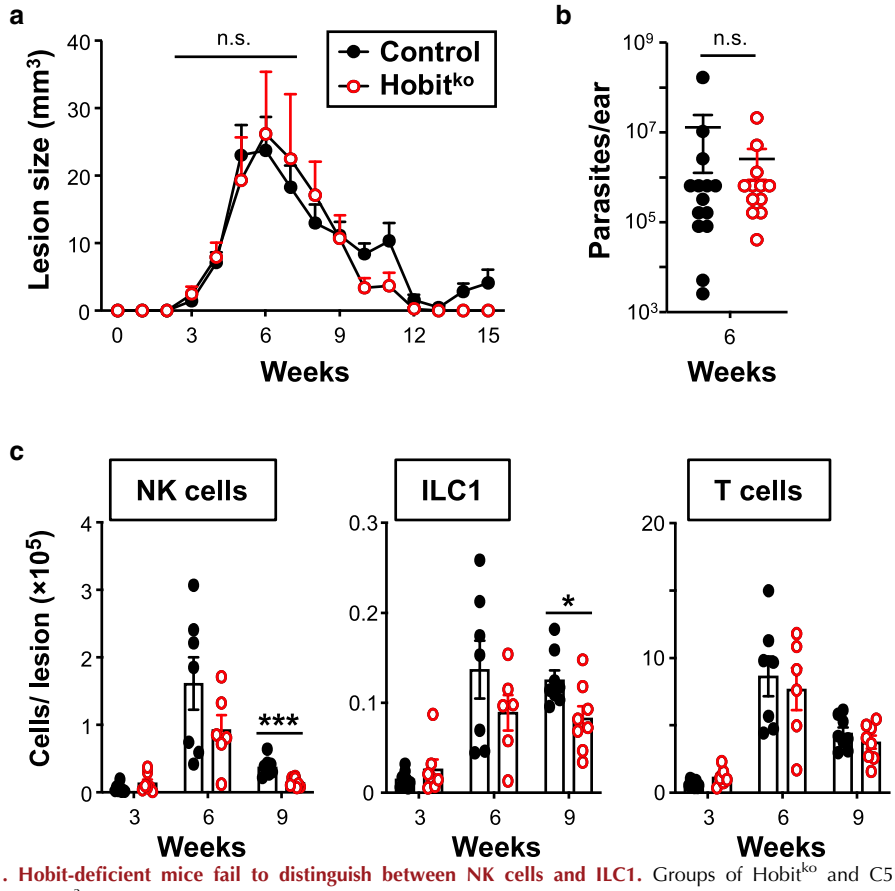

Supplementary Figure S1. Hobit-deficient mice fail to distinguish between NK cells and ILC1. Groups of Hobit^{ko} and C57BL/6 mice were infected intradermally into ear skin with 10^3 *L major* promastigotes. (**a**) Lesion volumes at indicated time points after low-dose *L major* infection (mean ± SEM, n = 6 mice per group; n = 4 independent experiments, significance was calculated using 2-way ANOVA). (**b**) In week 6, parasite burdens were determined using limiting dilution assays (n = 5−7 mice per group). Data are shown as individual burdens, means are indicated with a bar. Significance was calculated using the unpaired *t*-test. (**c**) Absolute numbers of NK cells, ILC1, and T cells in skin lesions were determined by flow cytometry as described earlier. NK cells and ILC1 cells were identified as described in Figure 3, NK1.1 instead of Nkp46 was used to discriminate cells, and T cells were gated as viable CD45^{pos} Lin^{pos} CD90.2^{pos} cells (mean ± SEM, pooled data of n = 3−8 mice per group and 1−2 independent experiments; significance was assessed using unpaired *t*-test; *$P \leq .05$ and ***$P \leq .001$). ILC, innate lymphoid cell; ns, not significant.