The Journal of Immunology

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J Immunol 2018; 200:1737-1745; Prepublished online 24 January 2018;
doi: 10.4049/jimmunol.1701597
http://www.jimmunol.org/content/200/5/1737

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

$CD8^+$ T Cells Lack Local Signals To Produce IFN-γ in the Skin during *Leishmania* Infection

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Resolution of leishmaniasis depends upon parasite control and limiting inflammation. $CD4^+$ Th1 cells are required to control parasites, whereas $CD8^+$ T cells play a dual role: they promote Th1 cell differentiation but can also increase inflammation at the site of infection as a consequence of cytolysis. Although $CD8^+$ T cells taken from leishmanial lesions are cytolytic, in this study, we showed that only a few $CD8^+$ T cells produced IFN-γ. Correspondingly, only low levels of IL-12 and/or IL-12 mRNA were present in lesions from infected mice, as well as patients. Addition of IL-12 increased IFN-γ production by $CD8^+$ T cells isolated from leishmanial lesions, suggesting that a lack of IL-12 at the site of infection limits IFN-γ production by $CD8^+$ T cells. To determine whether $CD8^+$ T cells could promote resistance in vivo if IL-12 was present, we administered IL-12 to *Leishmania*-infected RAG mice reconstituted with $CD8^+$ T cells. IL-12 treatment increased the ability of $CD8^+$ T cells to make IFN-γ, but $CD8^+$ T cells still failed to control the parasites. Furthermore, despite the ability of $CD8^+$ T cells to promote immunity to secondary infections, we also found that $CD8^+$ T cells from immune mice were unable to control *Leishmania* in RAG mice. Taken together, these results indicate that lesional $CD8^+$ T cells fail to make IFN-γ because of a deficit in IL-12 but that, even with IL-12, $CD8^+$ T cells are unable to control *Leishmania* in the absence of $CD4^+$ T cells. ***The Journal of Immunology*****, 2018, 200: 1737–1745.**

Cutaneous leishmaniasis is a major public health problem, with an estimate of one million new cases each year (1). Disease develops after infection with parasites belonging to the genus *Leishmania*, and the parasite species and the immune response of the infected host determine disease severity (2). Therefore, dissecting the role that the immune response plays in controlling disease or promoting inflammation is essential for designing vaccines and therapies for leishmaniasis patients.

Upon *Leishmania* infection, dendritic cells release the cytokine IL-12 and induce the differentiation of $CD4^+$ T cells into Th1 cells, a critical step for IFN-γ production (3, 4). The production of IFN-γ is essential to control *Leishmania* parasites through the generation of NO and superoxide anion, because both can effectively kill *Leishmania* parasites (5, 6). In addition to $CD4^+$ T cells, $CD8^+$ T cells are capable of making IFN-γ in leishmaniasis (7–10). In fact, IFN-γ produced by $CD8^+$ T cells contributes to $CD4^+$ T cell differentiation into protective Th1 cells postinfection (7). Conversely, $CD8^+$ T cells present in the skin can contribute to inflammation, thereby promoting disease severity in murine and human cutaneous leishmaniasis (11–17). The inability of $CD8^+$ T cells alone to play a protective role can be experimentally demonstrated by adoptively transferring $CD8^+$ T cells into RAG mice, which leads to severe pathology and no parasite control (10, 13). Once recruited into lesions, $CD8^+$ T cells exhibit a cytotoxic profile that results in killing of infected and uninfected cells, inflammasome activation, and IL-1β release (12). This cascade of events promotes severe inflammation and parasite dissemination and is associated with grave disease manifestations in patients. Therefore, $CD8^+$ T cells have been shown to play distinctive functions in disease: they can play a protective role by producing IFN-γ that promotes Th1 cell development, or they can be pathogenic in the skin by being cytotoxic.

Because $CD8^+$ T cells have been associated with promoting protection in low-dose primary infections (7, 10), as well as in resistance to secondary infections (8, 9), they have long been considered a target for a leishmanial vaccine (18–21). However, given their potential pathologic role, an important question to address is whether their cytolytic (and consequently pathologic) activity can be limited, thus generating $CD8^+$ T cells that only play a protective role. To address this, we adoptively transferred perforin-deficient $CD8^+$ T cells into RAG mice, which blocked the immunopathologic activity of $CD8^+$ T cells; however, $CD8^+$ T cells were still unable to control the parasites (13).

In this study, we have investigated whether the inability of $CD8^+$ T cells to provide protection in the absence of $CD4^+$ T cells might be due to a deficit in IFN-γ production by $CD8^+$ T cells at the infection site. We found that $CD8^+$ T cells do not make IFN-γ within lesions and that the inability of $CD8^+$ T cells to produce IFN-γ in the skin can be explained by the lack of local IL-12 production. This led us to test whether $CD8^+$ T cells could provide protection in the absence of $CD4^+$ T cells if they made IFN-γ. Exogenous administration of IL-12 induced IFN-γ–producing $CD8^+$ T cells in the skin; however, $CD8^+$ T cells were unable to provide protection in the absence of $CD4^+$ T cells. Immune $CD8^+$

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Received for publication November 20, 2017. Accepted for publication December 20, 2017.

This work was supported by National Institutes of Health/National Institute of Allergy and Infectious Diseases Grant R01 AI 106842.

The data presented in this article have been submitted to the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE55664.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BFA, brefeldin A; dLN, draining LN; KO, knockout; LN, lymph node; WT, wild-type.

T cells also could not prevent parasite replication; therefore, we conclude that $CD8^+$ T cells are pathogenic in the skin after *Leishmania* infection and cannot be rendered protective, even when signals to induce IFN-γ are provided.

Materials and Methods

Mice

This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee, University of Pennsylvania Animal Welfare. BALB/c and C57BL/6 CD45.2 or CD45.1 mice (6 wk old) were purchased from Charles River, and $RAG^{-/-}$ ($B6.129S7\text{-}RAG1^{tm1Mom}$) and C57BL/6 IL-12p40 YFP reporter mice were purchased from The Jackson Laboratory. *Ifng/Thy1.1* knock-in mice were provided by C. Weaver (University of Alabama). Both males and females were used for experiments. All mice were maintained in a specific pathogen–free environment at the University of Pennsylvania Animal Care Facilities.

Parasites

Leishmania braziliensis parasites (strain MHOM/BR/01/BA788) and *L. major* Friedlin strain (MHOM/IL/80/FN) were grown in Schneider's insect medium (Life Technologies), supplemented with 20% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Metacyclic-enriched promastigotes were used for infection (22). Mice were infected with 10^5 or 10^6 metacyclic promastigotes in the left ear, and the course of lesion progression was monitored weekly by measuring the diameter of ear induration with digital calipers (Fisher Scientific).

DNA and rIL-12 treatment

The DNA adjuvant construct encoding IL-12 has been described previously (23) and was provided and supplied by Inovio Pharmaceuticals. BALB/c mice were injected with 60 μg of IL-12 or empty plasmid in the ear, together with *Leishmania* parasites. RAG mice were infected and treated i.p. with 0.5 μg of rIL-12 every day until day 9 postinfection.

Cell purification and adoptive transfer

Splenocytes were collected from CD45.2 Thy1.1 IFN-γ reporter mice and labeled with 1.25 μM CFSE for 10 min at room temperature. The reaction was quenched by the addition of newborn calf serum, and cells were washed by centrifugation three times. Cell suspension was transferred i.v. into CD45.1 recipients that were immediately infected with *L. major*. For experiments with RAG mice, splenocytes from C57BL/6 mice were collected, RBCs were lysed with ACK lysing buffer (Lonza), and $CD8^+$ T cells were purified using a magnetic bead separation kit (Miltenyi Biotec). Three million $CD8^+$ T cells were transferred into RAG mice that were subsequently infected with *L. braziliensis*. Mice reconstituted with $CD8^+$ T cells received four injections of 250 μg of anti-CD4 within the first 2 wk to ensure that no $CD4^+$ T cells were present.

Skin preparation

Infected and uninfected ears were harvested, the dorsal and ventral layers of the ear were separated, and the ears were incubated in RPMI 1640 (Life Technologies) with 250 μg/ml Liberase TM (Roche) for 90 min at 37°C/5% CO_2. Following incubation, the enzyme reaction was stopped using 1 ml of RPMI 1640 medium containing 10% FBS. Ears were dissociated using a cell strainer (40 μm; BD Pharmingen), and an aliquot of the cell suspension was used for parasite titration.

In vitro stimulation of skin cells with cytokines

Skin cell suspension was incubated overnight with RPMI 1640, supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated with media only, or cytokines were added (IL-12 at 20 ng/ml and IL-18 at 50 ng/ml).

Parasite titration

The parasite burden in the ears was quantified as described previously (7). Briefly, the homogenate was serially diluted (1:10) in 96-well plates and incubated at 26°C. The number of viable parasites was calculated from the highest dilution at which parasites were observed after 7 d.

Flow cytometric analysis

Cell suspensions from mice were incubated with PMA (50 ng/ml), ionomycin (500 ng/ml), and brefeldin A (BFA; 10 μg /ml) (all from Sigma) or BFA only, as indicated, for IFN-γ intracellular staining. Before surface and intracellular staining, cells were washed and stained with a LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Molecular Probes), according to the manufacturer's instructions. All flow cytometry analyses were performed using FlowJo software. The following Abs were used: anti-CD11b eFluor 450, anti-CD3 eFluor 450, anti-Thy1.1 (CD90.1) PE-Cy7, anti–IFN-γ PE-Cy7 (all from eBioscience), anti-CD4 allophycocyanin–Alexa Fluor 780 (Invitrogen), and anti-CD8β PerCP-Cy5.5 (BioLegend).

Transcriptional profiling

For transcriptional profiling, cRNA was generated from 10 normal skin and 25 lesion biopsy samples, as described previously (14). Data have been submitted to the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE55664.

Statistical analysis

Data are presented as mean ± SE or individual samples. Statistical significance was determined using a two-tailed unpaired Student *t* test. All statistical analyses were calculated using Prism software (GraphPad). Differences were considered significant when $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

Results

$CD8^+$ T cells fail to produce IFN-γ in Leishmania*-infected skin*

IFN-γ is essential for controlling *Leishmania* infection (2) and although IFN-γ from $CD8^+$ T cells facilitates Th1 cell differentiation in the lymph nodes (LNs), the inability of $CD8^+$ T cells to provide protection in the absence of $CD4^+$ T cells might be because they fail to produce IFN-γ within lesions. To directly address this issue, $CD8^+$ T cells from the skin and draining LNs (dLNs) of *L. major*–infected C57BL/6 mice were analyzed for IFN-γ production after PMA and ionomycin stimulation. We found that $CD8^+$ T cells from the LNs were capable of producing IFN-γ, whereas $CD8^+$ T cells from the skin produced little to no IFN-γ (Fig. 1A, 1B). Without PMA and ionomycin stimulation, IFN-γ was not detected in the skin or LNs at this time point (data not shown).

We next compared IFN-γ expression in $CD4^+$ and $CD8^+$ T cells from the lesions and used an IFN-γ reporter mouse that expresses Thy1.1 as a result of IFN-γ transcription, thus allowing us to directly assess IFN-γ without restimulation of the cells (24). At 2 wk postinfection (Fig. 1C–F) and at 4 wk postinfection (Fig. 1G–J), $CD4^+$ T cells produced IFN-γ, whereas significantly fewer $CD8^+$ T cells present in the infected skin were capable of producing IFN-γ (Fig. 1C, 1D, 1G, 1H). Not only were there few $CD8^+$ T cells that produced IFN-γ, $CD8^+$ T cells also produced lower amounts of IFN-γ on a per-cell basis, as evidenced by lower mean fluorescence intensity levels (Fig. 1E, 1I). An analysis of the number of IFN-γ–producing cells indicated that more $CD4^+$ T cells produce IFN-γ in comparison with $CD8^+$ T cells (Fig. 1F, 1J), and when we phenotyped all of the cells within lesions that were making IFN-γ, >80% were $CD4^+$ T cells (Supplemental Fig. 1). To ensure that we were studying T cells that were responding to the infection, we labeled splenocytes with CFSE and transferred them to CD45 congenic mice (Fig. 2A). The majority of donor cells that we found in the lesions 2 wk later were $CFSE^{dim}$, indicating that they had proliferated in response to the infection. As above, we found that $CD4^+$ T cells were producing IFN-γ in the lesions, but proliferating $CD8^+$ T cells still produced very little IFN-γ (Fig. 2B, 2C). To determine whether this was unique to *L. major*, we assessed IFN-γ production by $CD8^+$ T cells in lesions from *L. braziliensis*–infected mice. C57BL/6 mice were infected with *L. major* (Supplemental Fig. 2A, 2B) or

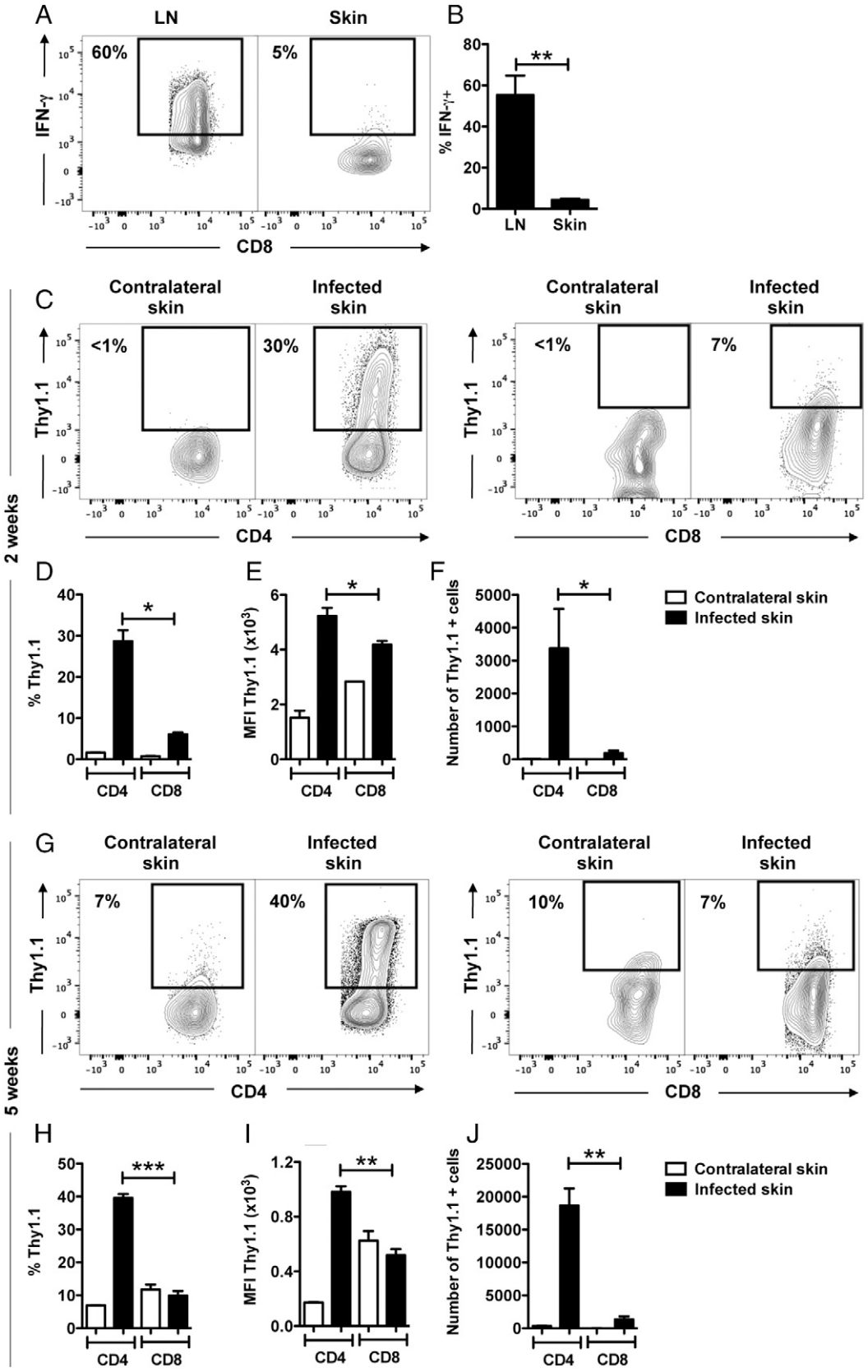

FIGURE 1. $CD8^+$ T cells do not produce IFN-γ in the skin in response to *L. major*. C57BL/6 mice were infected in the skin with 10^6 *L. major* and were euthanized 5 wk postinfection (**A** and **B**). The expression of intracellular IFN-γ in $CD8^+$ T cells was measured by flow cytometry in dLNs (LN) and infected ears. Representative contour plots (A) and bar graph (B) showing the percentage of IFN-γ–expressing $CD8^+$ T cells in dLNs (LN) and infected skin. IFN-γ reporter (Thy1.1) mice were infected in the skin with 10^6 *L. major*, and mice were euthanized 2 wk postinfection (**C**–**F**) or 4 wk postinfection (**G**–**J**). The expression of Thy1.1 directly ex vivo in $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry in contralateral and infected ears. Representative contour plots (C and G) and bar graphs showing the percentage (D and H), mean fluorescence intensity (MFI) (E and I), and number (F and J) of Thy1.1-expressing $CD4^+$ and $CD8^+$ T cells. Flow plots were pregated on live/singlets/CD3/CD8β or CD4. Representative data from three or more independent experiments (n = 3–5 mice per group) are presented. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

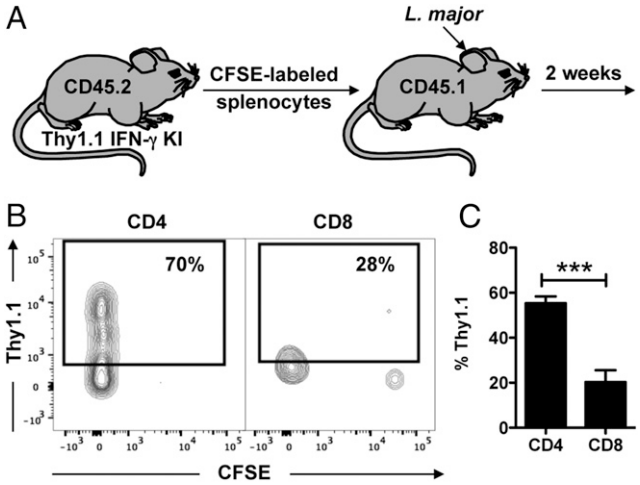

FIGURE 2. CD8$^+$ T cells that have proliferated in response to *L. major* infection do not produce IFN-γ in the skin. (**A**) Splenocytes from CD45.2 Thy1.1 IFN-γ reporter mice were stained with CFSE and transferred into CD45.1 congenic mice infected with *L. major*. Two weeks postinfection, mice were euthanized, and donor cells were analyzed for CFSE dilution and IFN-γ production. Representative contour plots (**B**) and bar graph (**C**) of Thy1.1-expressing donor CD4$^+$ and CD8$^+$ T cells. Flow plots were pregated on live/singlets/CD3/CD8β or CD4. Representative data from four independent experiments (n = 3 mice) are presented. ***$p \leq 0.001$.

L. braziliensis (Supplemental Fig. 2C, 2D) or BALB/c mice were infected with *L. braziliensis* (Supplemental Fig. 2E, 2F), and intracellular IFN-γ production without stimulation was determined by flow cytometry at 5 wk postinfection. Cells from the skin of all groups of infected mice showed a greater percentage of CD4$^+$ T cells expressing IFN-γ protein than CD8$^+$ T cells (Supplemental Fig. 2A–F). Taken together, these results show that CD8$^+$ T cells are not a major source of IFN-γ within leishmanial lesions.

IL-12 expression is deficient in Leishmania*-infected skin from mice and humans*

IL-12 induces IFN-γ production and is required for resistance to *Leishmania* infection (3, 25). Therefore, we considered the possibility that IL-12 levels in the skin were insufficient to induce IFN-γ production by CD8$^+$ T cells. We assessed expression of IL-12 in the lesions of *L. major*– or *L. braziliensis*–infected mice, for which we used an IL-12p40 reporter mouse. We found that IL-12p40 expression was not altered in mice infected with *L. major* or *L. braziliensis* in comparison with the contralateral ears (Fig. 3A, 3B). However, we could detect IL-12p40 expression in dLNs of the same groups of mice (Fig. 3C, 3D). The low-level expression of IL-12 mRNA was not only seen in mice. We previously published a genomic profiling comparing human normal skin with *L. braziliensis* patient lesions (14), and we used that dataset in this study to determine whether IL-12 was differentially expressed between the two groups. We found that *IL12A* and *IL12B* mRNA levels are unchanged between normal skin and lesions (Fig. 3E, 3F). Importantly, the levels of expression in both groups reached the lower limit of detection of the assay, suggesting that *IL12A* and *IL12B* are not expressed in the skin of normal or lesion biopsies. Together, these results indicate that IL-12 is not present in the skin of patients or mice infected with *Leishmania*; this led us to hypothesize that CD8$^+$ T cells are defective in IFN-γ production due to the lack of IL-12 signaling at the site of infection.

CD8$^+$ T cells from leishmanial lesions make IFN-γ in the presence of IL-12

To determine whether CD8$^+$ T cells from lesions could make IFN-γ if IL-12 were present, single-cell suspensions from the

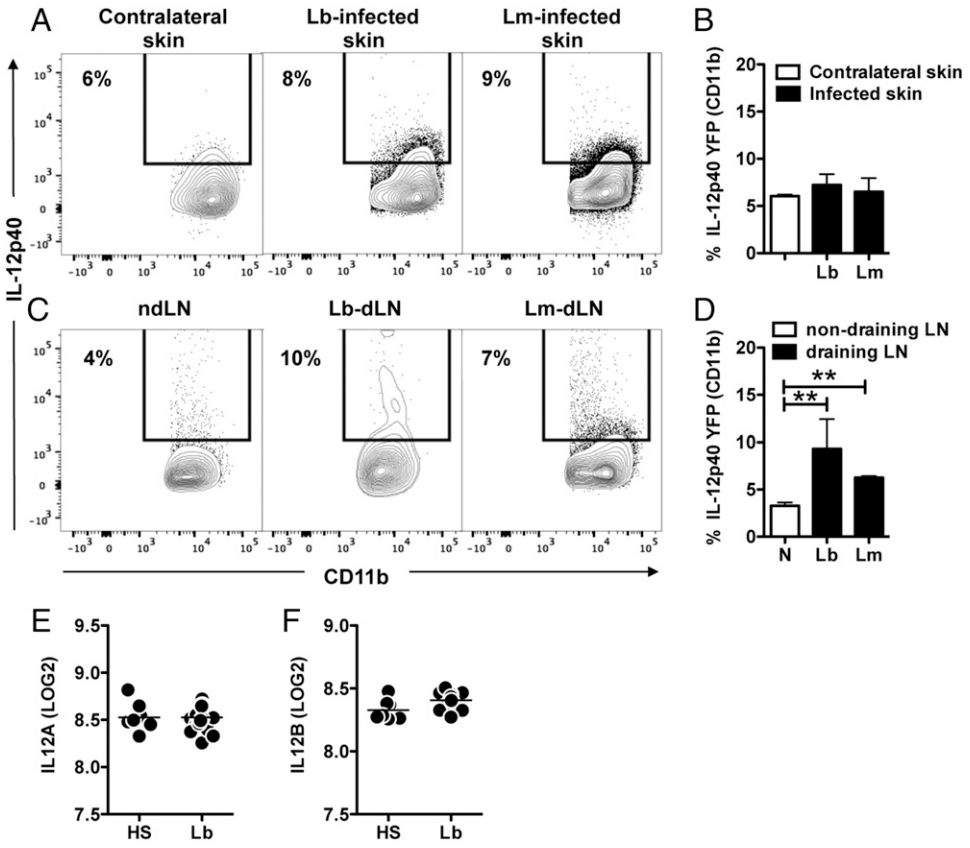

FIGURE 3. IL-12 is not produced in *Leishmania* lesions from mice and humans. IL-12p40 reporter mice were infected in the skin with *L. major* or *L. braziliensis* and were euthanized 2 wk postinfection. Cells from the contralateral (a combination of contralateral skin from *L. braziliensis*– and *L. major*–infected mice) and infected skin (**A** and **B**) or from nondraining (a combination of nondraining LNs from *L. braziliensis*– and *L. major*–infected mice) and dLNs (**C** and **D**) were analyzed for IL-12p40 expression directly ex vivo by flow cytometry. Representative contour plots (A and C) and bar graphs (B and D) showing the percentage of IL-12p40$^+$ CD11b$^+$ cells. Flow plots were pregated on live/singlets/CD11b. Representative data from two independent experiments (n = 3 mice per group) with similar results are presented. Log2 expression of *IL12A* (**E**) and *IL12B* (**F**) in the skin of healthy subjects (HS; n = 10) and *L. braziliensis* patients (Lb; n = 25). **$p \leq 0.01$.

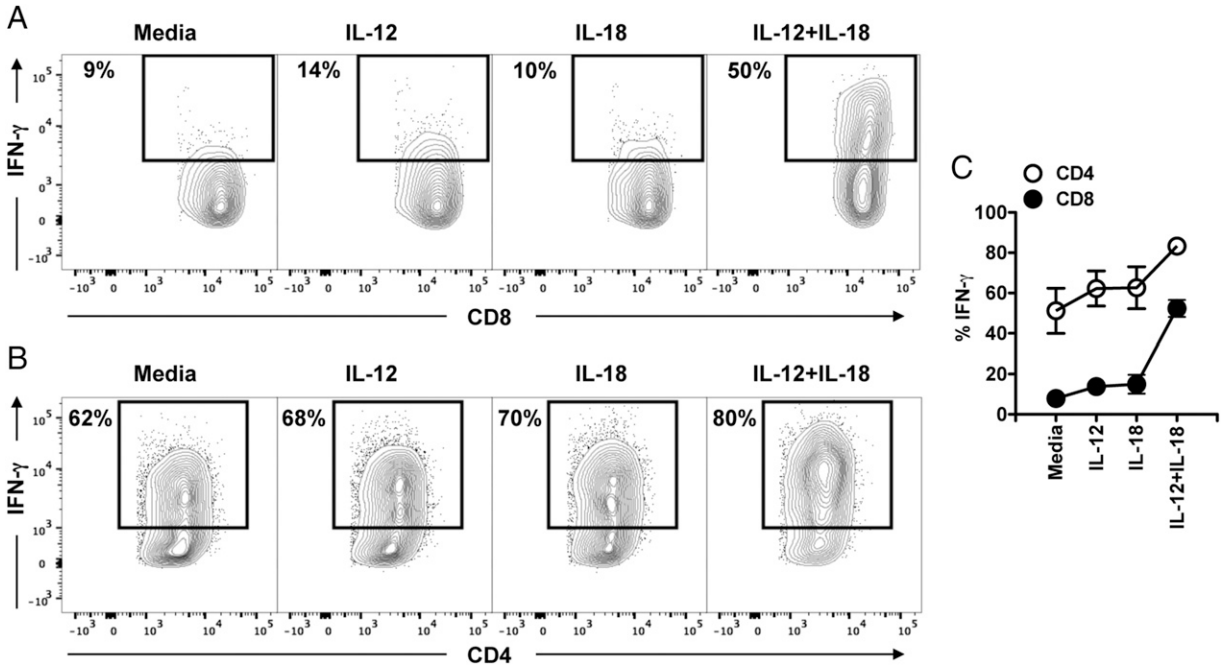

FIGURE 4. $CD4^+$ and $CD8^+$ T cells have different requirements for IFN-γ in *Leishmania*-infected skin. C57BL/6 mice were infected in the skin with 10^6 *L. major* and were euthanized 2 wk postinfection. Cells from infected skin were cultured with media or cytokines overnight (with BFA for the last 4 h), and the expression of IFN-γ was measured by flow cytometry in $CD4^+$ and $CD8^+$ T cells. Representative contour plots (**A** and **B**) and line graph (**C**) showing IFN-γ. Representative data from four independent experiments (n = 3 mice per group) with similar results are presented.

lesions of mice infected with *L. major* for 2 wk were incubated or not with IL-12 overnight (the cells received BFA for the last 4 h of culture), and IFN-γ intracellular protein expression was determined by flow cytometry. Cultures without the addition of cytokines (media) showed that $CD8^+$ T cells did not make IFN-γ (Fig. 4A), whereas an average of 60% of $CD4^+$ T cells present in the skin produced IFN-γ (Fig. 4B). Although IL-12 or IL-18 alone did not significantly alter IFN-γ production by $CD4^+$ or $CD8^+$ T cells (Fig. 4A, 4B), $CD4^+$ and $CD8^+$ T cells stimulated with IL-12 in the presence of IL-18 had increased IFN-γ production (Fig. 4C). Although $CD8^+$ T cells had a significantly increased capacity to make IFN-γ in the presence of IL-12 + IL-18, only 50% of those cells produced IFN-γ in this ideal scenario. These results indicate that a lack of IL-12 in the skin has a dramatic impact on the capacity of $CD8^+$ T cells to produce IFN-γ, whereas it has only a mild effect on IFN-γ production by $CD4^+$ T cells.

IL-12 treatment in vivo enhances IFN-γ production by $CD8^+$ T cells in the skin

To determine whether providing IL-12 in the skin early postinfection could enhance IFN-γ production by $CD8^+$ T cells, we infected mice with *L. braziliensis* in the presence of an IL-12 plasmid or a control plasmid. As expected, administration of the IL-12 plasmid at the site of infection reduced lesion development in mice and significantly decreased the number of parasites in the skin 6 wk postinfection (Fig. 5A, 5B). To test whether IL-12 administration had an impact on IFN-γ production by $CD8^+$ T cells,

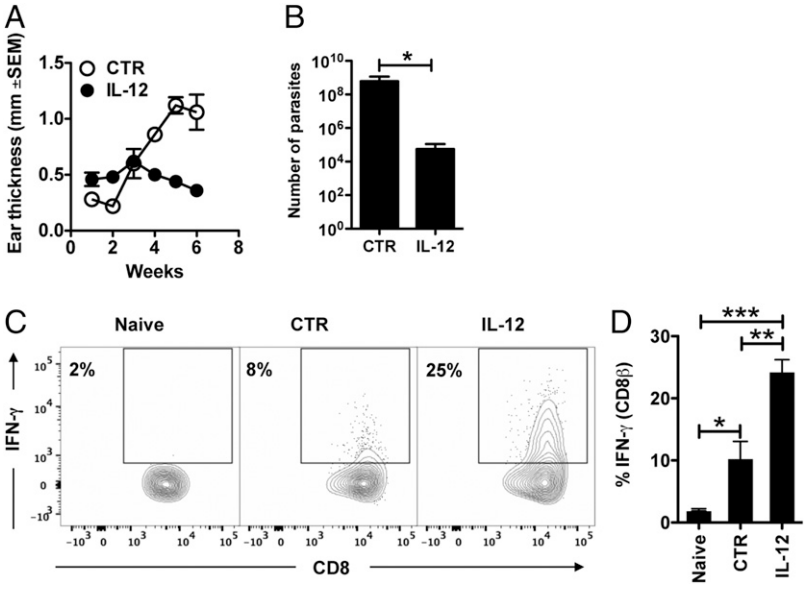

FIGURE 5. IL-12 treatment enhances IFN-γ production by $CD8^+$ T cells in the skin. (**A**) BALB/c mice were infected in the skin with 10^5 *L. braziliensis* in conjunction with a control (CTR) or IL-12 plasmid, and ear thickness was assessed weekly. (**B**) Mice were euthanized 6 wk postinfection, the number of parasites was determined in the skin, and lesions were digested and used for flow cytometric analysis of intracellular IFN-γ. Representative contour plots (**C**) and bar graph (**D**) of IFN-γ intracellular staining. Flow plots were pregated on live/singlets/CD3/CD8β. Data from one experiment (n = 5 mice per group) are shown. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

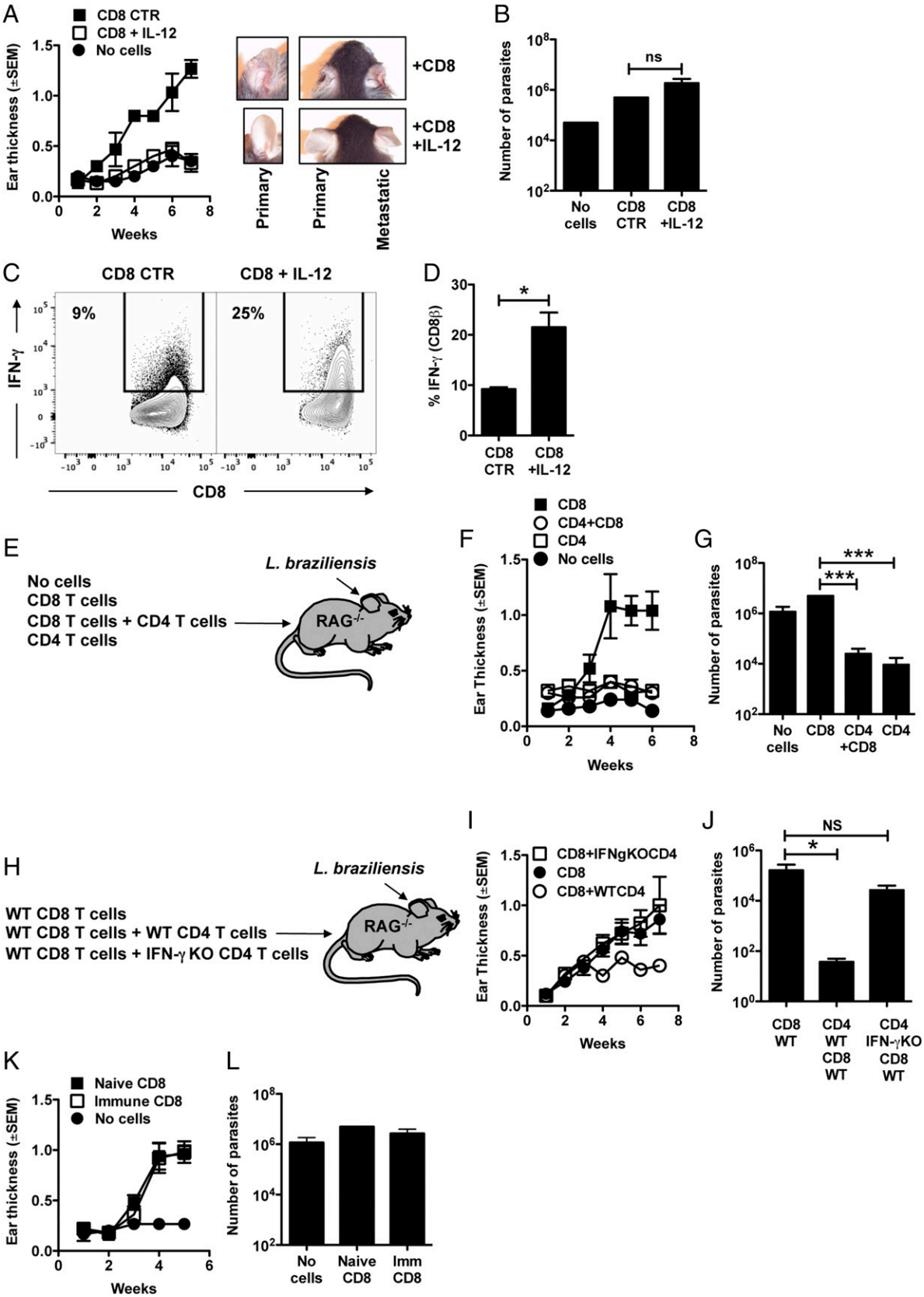

FIGURE 6. $CD8^+$ T cells are unable to control *Leishmania* infection. $RAG^{-/-}$ mice were infected with *L. braziliensis* and were reconstituted or not with $CD8^+$ T cells (**A**–**D**). Mice were treated i.p. with 0.5 mg of IL-12 on days 0–9. The course of infection (A) and number of parasites (B) were assessed in the skin at 7 wk postinfection. Representative contour plots (C) and bar graphs (D) of IFN-γ expression in $CD8^+$ T cells from the skin. Representative data from four independent experiments (n = 3–5 mice per group) with similar results are presented. Flow plots were pregated on live/singlets/CD3/CD8β. (**E**) $RAG^{-/-}$ mice were infected with *L. braziliensis* and reconstituted or not with $CD8^+$ T cells, $CD4^+$ T cells, or $CD8^+$ and $CD4^+$ T cells. The course of infection (**F**) and number of parasites (**G**) were assessed in the skin at 7 wk postinfection. Representative data from two independent experiments (n = 5 mice per group) with similar results are presented. (**H**) $RAG^{-/-}$ mice were infected with *L. braziliensis* and reconstituted or not with WT *(Figure legend continues)*

we created single-cell suspensions from the ears of naive, control, or IL-12–treated mice and assessed intracellular IFN-γ production by $CD8^+$ T cells by flow cytometry. We found that mice that received IL-12 had a significantly higher percentage of $CD8^+$ T cells producing IFN-γ in the skin after PMA and ionomycin stimulation directly ex vivo (Fig. 5C, 5D). Hence, IL-12 administration in the skin provides signals to induce IFN-γ–producing $CD8^+$ T cells in the skin.

IL-12 administration is not sufficient to induce protection by $CD8^+$ T cells in vivo

To determine whether IL-12 administration could promote the development of IFN-γ–producing $CD8^+$ T cells that could mediate protection in the absence of $CD4^+$ T cells, RAG mice were left untreated or were infected with *L. braziliensis*, reconstituted with $CD8^+$ T cells, and given injections of rIL-12 during the first week of infection. RAG mice reconstituted with $CD8^+$ T cells and infected with *L. major* or *L. braziliensis* develop severe nonhealing lesions without any evidence of parasite control (10, 13). We hypothesized that IL-12 administration would promote the development of protective $CD8^+$ T cells and that a reduced parasite burden would limit pathology. Indeed, we found that administration of rIL-12 to mice prevented the development of severe lesions in those that received wild-type (WT) $CD8^+$ T cells (Fig. 6A, left panel). Treatment with IL-12 prevented the development of severe lesions at the primary site, and it blocked the development of metastatic lesions at other skin sites (Fig. 6A, right panels). Surprisingly, the abrogated lesion development in mice treated with IL-12 was not accompanied by a reduction in the number of parasites in the skin (Fig. 6B), although we could detect an increase in IFN-γ–expressing $CD8^+$ T cells in the skin of mice after treatment with IL-12 (Fig. 6C, 6D). These results indicate that, although IL-12 plays a role in regulating the $CD8^+$ T cell response in RAG mice, $CD8^+$ T cells by themselves, even in the presence of IL-12, are unable to provide parasite control.

IFN-γ derived from $CD4^+$ T cells is sufficient for parasite control in Leishmania *infection*

To determine whether $CD4^+$ T cells alone were capable of controlling parasites, we infected RAG mice with *L. braziliensis* in the skin and reconstituted mice with $CD8^+$ T cells alone, $CD8^+$ T cells and $CD4^+$ T cells, $CD4^+$ T cells alone, or no cells (Fig. 6E). As previously demonstrated (13), RAG mice with no cells and those that were reconstituted with $CD8^+$ T cells have similar numbers of parasites in the skin, although lesions are nearly absent in RAG mice with no cells and are large and severe in RAG mice with $CD8^+$ T cells (Fig. 6F, 6G). In contrast, RAG mice that were reconstituted with $CD4^+$ T cells and $CD8^+$ T cells control lesion development and parasite growth (Fig. 6F, 6G) (13). Importantly, mice reconstituted with $CD4^+$ T cells alone and the combination of $CD4^+$ T cells and $CD8^+$ T cells control parasites, suggesting that $CD4^+$ T cells alone can control *L. braziliensis* infection (Fig. 6F, 6G). To test whether this is due to IFN-γ production, we reconstituted RAG mice with WT $CD8^+$ T cells together with WT or IFN-γ–deficient (IFN-γ–knockout [KO]) $CD4^+$ T cells (Fig. 6H). Our results showed that RAG mice reconstituted with IFN-γ–KO $CD4^+$ T cells + WT $CD8^+$ T cells had similar lesion sizes and parasite numbers compared with mice reconstituted with $CD8^+$ T cells alone, and they had significantly larger lesions and higher parasite numbers than RAG mice reconstituted with WT $CD4^+$ T cells + $CD8^+$ T cells (Fig. 6I, 6J). Together, these results suggest that IFN-γ derived from $CD4^+$ T cells is not only required, but is also sufficient, to control *Leishmania* parasites in the skin.

$CD8^+$ T cells from immune mice fail to provide protection in RAG mice

$CD8^+$ T cells have long been considered targets for vaccine development in leishmaniasis. Hence, we next asked whether immune $CD8^+$ T cells were better able to provide protection than effector $CD8^+$ T cells. RAG mice were infected with *L. braziliensis* and reconstituted with $CD8^+$ T cells obtained from naive or immune mice. The course of infection (Fig. 6K) and parasite numbers in the skin (Fig. 6L) showed that, similar to naive $CD8^+$ T cells, immune $CD8^+$ T cells were unable to protect RAG mice from *L. braziliensis* infection. These data suggest that even primed $CD8^+$ T cells from immune mice by themselves are unable to control *L. braziliensis* infection.

Altogether, our results demonstrate that the skin microenvironment of *Leishmania*-infected mice is deficient in the appropriate signals to promote IFN-γ production by $CD8^+$ T cells. The lack of sufficient IL-12 in the skin prevents $CD8^+$ T cells from becoming IFN-γ producers without affecting $CD4^+$ Th1 cells, suggesting different requirements for $CD4^+$ and $CD8^+$ T cells in IFN-γ production. However, even in optimal conditions, in which IL-12 is provided, $CD8^+$ T cells are unable to provide protection.

Discussion

Leishmania parasites are controlled by IFN-γ that activates macrophages to kill intracellular parasites. It is well established that IL-12–dependent generation of IFN-γ–producing $CD4^+$ Th1 cells is critical for resistance to these parasites and that $CD8^+$ T cells can promote $CD4^+$ Th1 cell development, as well as enhance resistance to reinfection (3, 4, 7–9, 25, 26). Paradoxically, however, $CD8^+$ T cells have also been shown to mediate excessive inflammation in mice and in patients, promoting the destruction of the skin architecture and leading to ulcer development, as well as promoting more severe forms of the disease, such as mucosal and disseminated leishmaniasis (11–17, 27–29). Previous studies found that, in the absence of $CD4^+$ T cells, $CD8^+$ T cells were unable to control *Leishmania* infection (10, 13), and we investigated why this was the case in this study. We found that, in contrast to $CD8^+$ T cells in dLNs, $CD8^+$ T cells in *Leishmania* lesions fail to make IFN-γ. We discovered that the inability of $CD8^+$ T cells to make IFN-γ in lesions is due to a deficit in IL-12 production. Importantly, however, even when we administered IL-12 and increased the production of IFN-γ by $CD8^+$ T cells, the IFN-γ production was still insufficient to provide protection. Thus, our results indicate that the protective role for $CD8^+$ T cells is dependent upon the presence of $CD4^+$ T cells, and, in the absence of $CD4^+$ T cells, the primary role of $CD8^+$ T cells is pathologic.

$CD8^+$ T cells, WT $CD8^+$ and WT $CD4^+$ T cells, or WT $CD8^+$ and IFN-γ KO $CD4^+$ T cells. The course of infection (**I**) and number of parasites (**J**) were assessed in the skin at 7 wk postinfection. C57BL/6 mice were infected or not with *L. braziliensis* for 10–15 wk and were euthanized, and splenocytes were used as donors of $CD8^+$ T cells (**K** and **L**). $RAG^{-/-}$ mice were infected with *L. braziliensis* and reconstituted or not with immune or naive $CD8^+$ T cells. The course of infection (K) and number of parasites (L) were assessed in the skin at 7 wk postinfection. Representative data from three independent experiments with similar results are presented for the course of infection, and one experiment for parasite titration ($n = 5$ mice per group) $^*p \leq 0.05$, $^{***}p \leq 0.001$. ns, nonsignificant.

The heterogeneity of $CD8^+$ T cells is most often investigated in the context of longevity. For example, a large number of studies have described the characteristics of memory $CD8^+$ T cells and $CD8^+$ T cells that have the potential to develop into memory T cells (30). $CD8^+$ T cells can make cytokines (such as IFN-γ) and can be cytolytic, and it is often assumed that, once $CD8^+$ T cells become fully activated, they perform both functions; however, it is increasingly clear that this is not the case. Indeed, studies of $CD8^+$ T cell clones from HIV patients found that most $CD8^+$ T cells were cytolytic or made IFN-γ, and only a few performed both functions (31). The factors that lead to $CD8^+$ T cells exhibiting exclusively one function or the other are not particularly well understood, and this study indicates that one such factor may be the location of the $CD8^+$ T cells. We previously found that $CD8^+$ T cells in leishmanial lesions express granzymes and perforin, and they exhibit cytolytic activity that leads to extensive cell death, inflammation, IL-1β secretion, and pathology (13, 14). These results are consistent with previous findings showing that $CD8^+$ T cells within lymphoid tissues are defective at killing, whereas they are armed to kill target cells once in the tissues (32–34). In this article, we show that lesional $CD8^+$ T cells are pathologic and that they fail to make IFN-γ in the lesions as a result of limited IL-12 levels therein.

In contrast to the clear protective role that $CD8^+$ T cells play in visceral leishmaniasis, the role of $CD8^+$ T cells in protecting against cutaneous leishmaniasis is still poorly understood (35, 36). Although $CD8^+$ T cells were initially found not to be required for protection against *L. major* in mice (37–39), other studies found that they were required (10). We found that this discrepancy was due to a difference in the dose of parasites used to infect mice (7). $CD8^+$ T cells were not required for protection against a high dose of *L. major* parasites but were required when mice were infected with a low dose of parasites (7). The protective role for $CD8^+$ T cells in this model was to provide IFN-γ to promote $CD4^+$ Th1 cell development, because $CD8^+$-deficient mice infected with a low dose developed a dominant $CD4^+$ Th2 response (7). Thus, we hypothesized that one important function for $CD8^+$ T cells in leishmaniasis was to promote $CD4^+$ Th1 cell development in the dLN when there was a low level of Ag stimulation. Our current results indicate that this may be the only scenario in which $CD8^+$ T cells produce IFN-γ in cutaneous leishmaniasis.

The limited production of IL-12 within leishmanial lesions was unexpected, because IL-12 is required for the development of a protective immune response to *Leishmania* (3, 25). In contrast, *Leishmania* is not a strong inducer of IL-12, and several studies have shown that infected dendritic cells are unable to make IL-12, even when stimulated with LPS (40, 41). However, it should be pointed out that the literature on this issue is contradictory; this may be due to differences in parasites and/or host cells, as well as the assumption that IL-12 in a culture with infected and uninfected cells is originating from the infected cells (42–48). It has been shown that *Leishmania* infection inhibits IL-12 promoter activity, and this inhibition appears to be due to the use of CR3 for entry (41, 49). In fact, instead of the infected cells making IL-12, it is produced by uninfected bystander dendritic cells, which may be one reason why we fail to see high levels of IL-12 in lesions with lots of parasites (40). Although the mechanism involved in bystander production of IL-12 is not totally clear, we reported previously that a parasite product and TNF are required for bystander production of IL-12 (40). Interestingly, the deficit is limited to the lesion site, because IL-12 was present in dLNs. This might be anticipated, because LNs will contain the highest percentage of mature dendritic cells, with a lower number of infected cells.

Our data corroborate results from other groups showing that, in *Leishmania* patients, $CD4^+$ T cells produce more IFN-γ than $CD8^+$ T cells and that $CD4^+$ T cells are better able to control *Leishmania* infection in vitro compared with $CD8^+$ T cells (15). We expanded these findings in this study by demonstrating that $CD4^+$ T cells are the major source of IFN-γ, taking into account all other possible sources of IFN-γ, as well as that $CD4^+$ T cells are sufficient to control parasite replication and lesion development in cutaneous disease. One surprising finding was that $CD4^+$ T cells and $CD8^+$ T cells have distinct requirements for IFN-γ production. We previously reported that $CD4^+$ Th1 cells do not require IL-12 production to maintain a Th1 phenotype (50, 51), which is consistent with our current finding that $CD4^+$ T cells in the skin still express IFN-γ in the absence of IL-12. In contrast, we found that $CD8^+$ T cells in the skin need constant IL-12 signaling to produce IFN-γ during infection. Thus, $CD8^+$ T cells in leishmanial lesions appear to require a constant reminder in the form of IL-12 to make IFN-γ.

$CD8^+$ T cells have long been considered a good target for vaccination in *Leishmania* infection, and this may be the case for visceral leishmaniasis (35). However, based upon our results, we hypothesize that the protective role of $CD8^+$ T cells in cutaneous leishmaniasis is primarily an indirect effect and is mediated by their capacity to promote $CD4^+$ Th1 cell development in the dLN during priming, rather than their ability to control parasites within the skin. Thus, $CD8^+$ T cells may not be a great target for a vaccine in cutaneous leishmaniasis, because it is unclear how one would ensure that they made IFN-γ and were protective, rather than acting as cytolytic cells and promoting pathology.

Acknowledgments

We thank Ba Nguyen for assistance.

Disclosures

The authors have no financial conflicts of interest.

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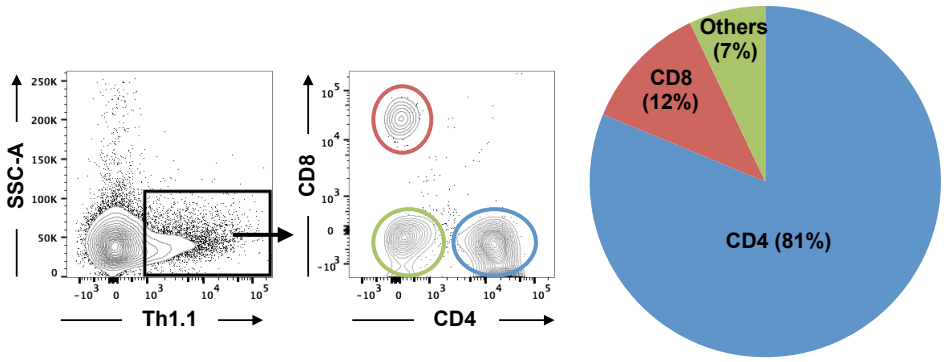
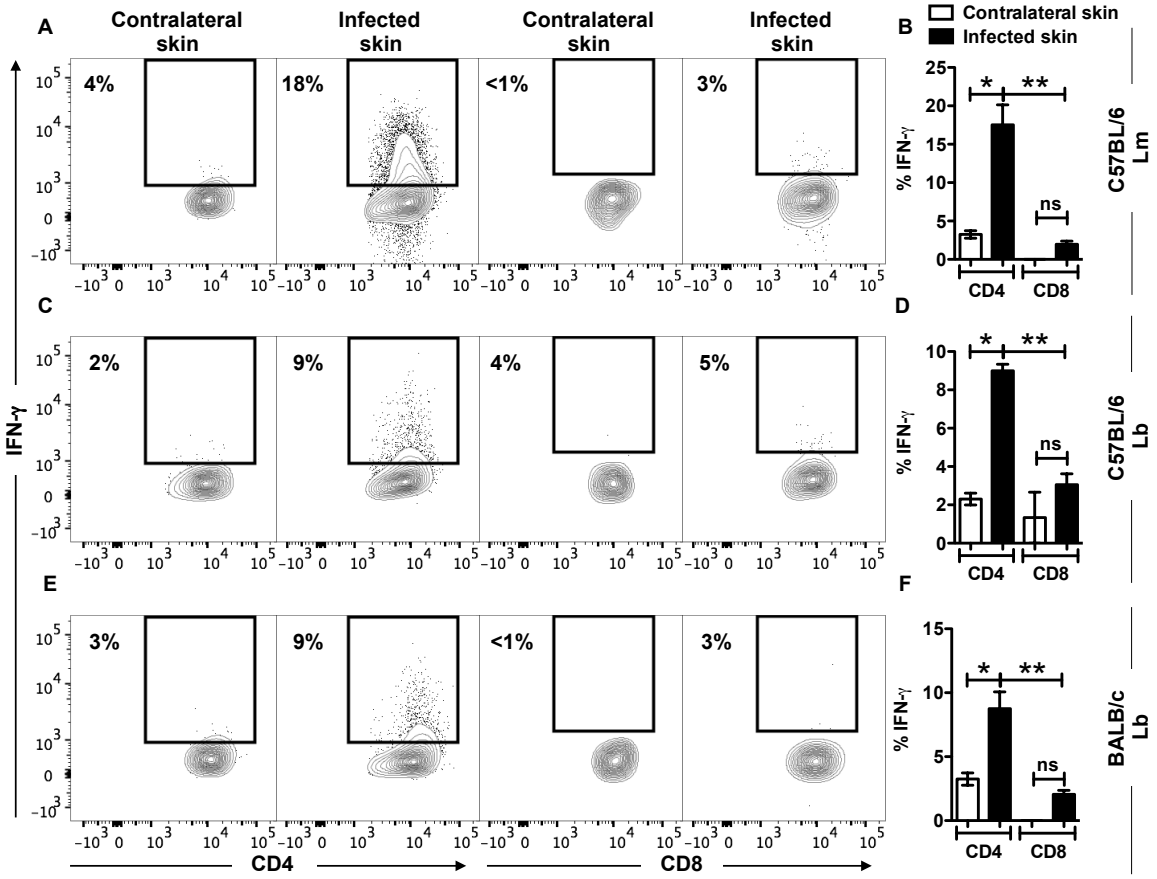


Supplemental Figure 1: CD4+ T cells are the major source of IFN-γ. Thy1.1 IFN-γ reporter mice were infected in the skin with 10^6 *L. major* and 2 weeks post infection mice were euthanized. Thy1.1 positive cells cells were checked for the expression of CD4+ and CD8+ T cell surface markers (representative flow plots, left) and percentages are represented in a pie chart (right) (n = 4 mice). Flow plots pregated on live/singlets/Thy1.1/CD8β or CD4.



Supplemental Figure 2: CD8+ T cells do not produce IFN-γ protein in the skin in response to *L.major* and *L. braziliensis*. C57BL/6 or BALB/c mice were infected in the skin with 10^6 *L. major* or 10^5 *L. braziliensis* and 5 weeks post infection mice were euthanized. Cells from the contralateral and infected skin were cultured for 6 hours in BFA and the expression of IFN-γ in CD4+ and CD8+ T cells was measured by flow cytometry. Depicted are (**A, C and E**) representative contour plots and (**B, D and F**) bar graphs showing the percentage of IFN-γ expressing CD4+ and CD8+ T cells. (**A and B**) C57BL/6 infected with *L. major*. (**C and D**) C57BL/6 infected with *L. braziliensis*. (**E and F**) BALB/c infected with *L. braziliensis*. Flow plots pregated on live/singlets/CD3/CD8β or CD4. Representative data from 3 or more independent experiments (n = 5 mice per group) with similar results are presented. *$p \leq 0.05$ or **$p \leq 0.01$; ns, non-significant