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RESEARCH ARTICLE

CCR5 promotes the migration of pathological CD8$^+$ T cells to the leishmanial lesions

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Abstract

Cytolytic CD8$^+$ T cells mediate immunopathology in cutaneous leishmaniasis without controlling parasites. Here, we identify factors involved in CD8$^+$ T cell migration to the lesion that could be targeted to ameliorate disease severity. CCR5 was the most highly expressed chemokine receptor in patient lesions, and the high expression of CCL3 and CCL4, CCR5 ligands, was associated with delayed healing of lesions. To test the requirement for CCR5, *Leishmania*-infected Rag1$^{-/-}$ mice were reconstituted with CCR5$^{-/-}$ CD8$^+$ T cells. We found that these mice developed smaller lesions accompanied by a reduction in CD8$^+$ T cell numbers compared to controls. We confirmed these findings by showing that the inhibition of CCR5 with maraviroc, a selective inhibitor of CCR5, reduced lesion development without affecting the parasite burden. Together, these results reveal that CD8$^+$ T cells migrate to leishmanial lesions in a CCR5-dependent manner and that blocking CCR5 prevents CD8$^+$ T cell-mediated pathology.

Author summary

Cutaneous leishmaniasis remains a serious disease in many parts of the world, and the current treatment is often ineffective. The increased pathology observed in some patients results from pathologic CD8$^+$ T cells, which promote the lysis of infected cells, leading to tissue destruction without controlling the parasites. Our objective is to develop host-directed therapies that could augment disease resolution. Here, we identify factors involved in CD8$^+$ T cell migration to cutaneous leishmaniasis lesions to reveal additional targets that could be used as host-directed therapies in patients. We found that CD8$^+$ T cells migrate to leishmanial lesions in a CCR5-dependent manner, and by blocking CD8$^+$ T cell migration with maraviroc, an FDA-approved selective inhibitor of CCR5, we decreased disease severity. Thus, we suggest that inhibiting CCR5 by maraviroc may be a useful approach to lessen disease in cutaneous leishmaniasis and other diseases where CD8$^+$ T cell mediates pathology.

publication. The complete RNA-seq data analysis, R code, file inputs, and outputs used to perform the transcriptional analysis presented in this current manuscript are available on the GitHub repository "CCR5_LAS" (https://github.com/camilafarias112/CCR5_LAS).

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Introduction

Cutaneous leishmaniasis is caused by an intracellular protozoan parasite transmitted by sandflies. The disease exhibits a broad spectrum of clinical manifestations ranging from self-healing lesions to extensive mucosal damage. While some patients resolve their lesions spontaneously, others develop lesions that progress to chronicity and lead to the development of severe mucosal disease. Importantly, the severe disease in many patients is due to the inflammatory response rather than uncontrolled parasite replication [1–5]. Thus, treatment of cutaneous leishmaniasis may require not only anti-parasitic drugs but also host-directed therapies to limit inflammation.

Cytolytic $CD8^+$ T cells play a pathological role in cutaneous leishmaniasis and contribute to the chronicity of the disease. Experimental murine models and transcriptional studies in patients' lesions demonstrate that cytolytic $CD8^+$ T cells mediate increased pathology by promoting extensive cytolysis, leading to inflammasome activation and interleukin-1β (IL-1β) release, which in turn feeds the inflammation and enhances the magnitude of the disease [6–10]. In previous studies, we found that inhibition of cytotoxicity, inflammasome activation or IL-1β release, blocked severe disease [6,10,11]. Since $CD8^+$ T cells initiate this pathway, defining the factors facilitating CD8+ T cell migration to leishmanial lesions is important and could identify additional therapeutic approaches to improve the treatment of the disease.

We found that patients with a high expression of *CCL3* and *CCL4*, which binds to the chemokine receptor CCR5, exhibited delayed healing of lesions and had elevated expression of key cytolytic genes. Translating the findings to the murine model, we found that $CD8^+$ T cells express CCR5 preferentially at the lesions and that deletion of CCR5 in $CD8^+$ T cells dampens severe pathology in a model of cutaneous leishmaniasis. This led to our finding that maraviroc (MVC), a selective inhibitor of CCR5, significantly reduced disease in our experimental model. Collectively, these results show that CCR5 mediates the migration of $CD8^+$ T cells to leishmanial lesions and identifies possible target for host-directed therapy.

Results

Genes encoding for CCR5 and its ligands are overexpressed in patient lesions and are associated with delayed healing

We previously demonstrated that cytotoxic $CD8^+$ T cells mediate the development of severe lesions in cutaneous leishmaniasis [7,8]. In order to characterize what drives the migration of $CD8^+$ T cells to lesions, we created a list of chemokine receptors associated with T cell migration based on the literature [12–19]. We took advantage of our published human RNA-seq dataset of lesions from *L. braziliensis* patients [7] and evaluated the expression of chemokine receptors and their ligands in biopsies from patients' lesions compared to healthy skin (Fig 1A). The analysis showed that among the chemokine receptors studied, *CCR5* was the most differentially expressed in lesions compared to healthy skin, and *CCL3* and *CCL4*, which bind CCR5, were also enriched in lesions (Fig 1B). *CCL3* and *CCL4* are statistically correlated in *L. braziliensis*-lesions ($^{**}p = 0.0049$), so we stratified the cohort of patients in half (normal distributions) based on the high ($CCL3/4^{high}$) or low ($CCL3/4^{low}$) expression of these genes (Fig 1C) and used this strategy to evaluate the impact on the healing time.

As demonstrated in Fig 1D, patients with $CCL3/4^{high}$ expression exhibited delayed healing compared to $CCL3/4^{low}$ patients. We analyzed the expression of *CCL3* and *CCL4* based on the patients that cured the lesions at 90 days or after 90 days, <90d or >90d, respectively. Reinforcing our findings, we observed that patients who did not resolve the lesions and needed more rounds of treatment had a higher expression of *CCL3* and *CCL4* at the lesions (S1 Fig).

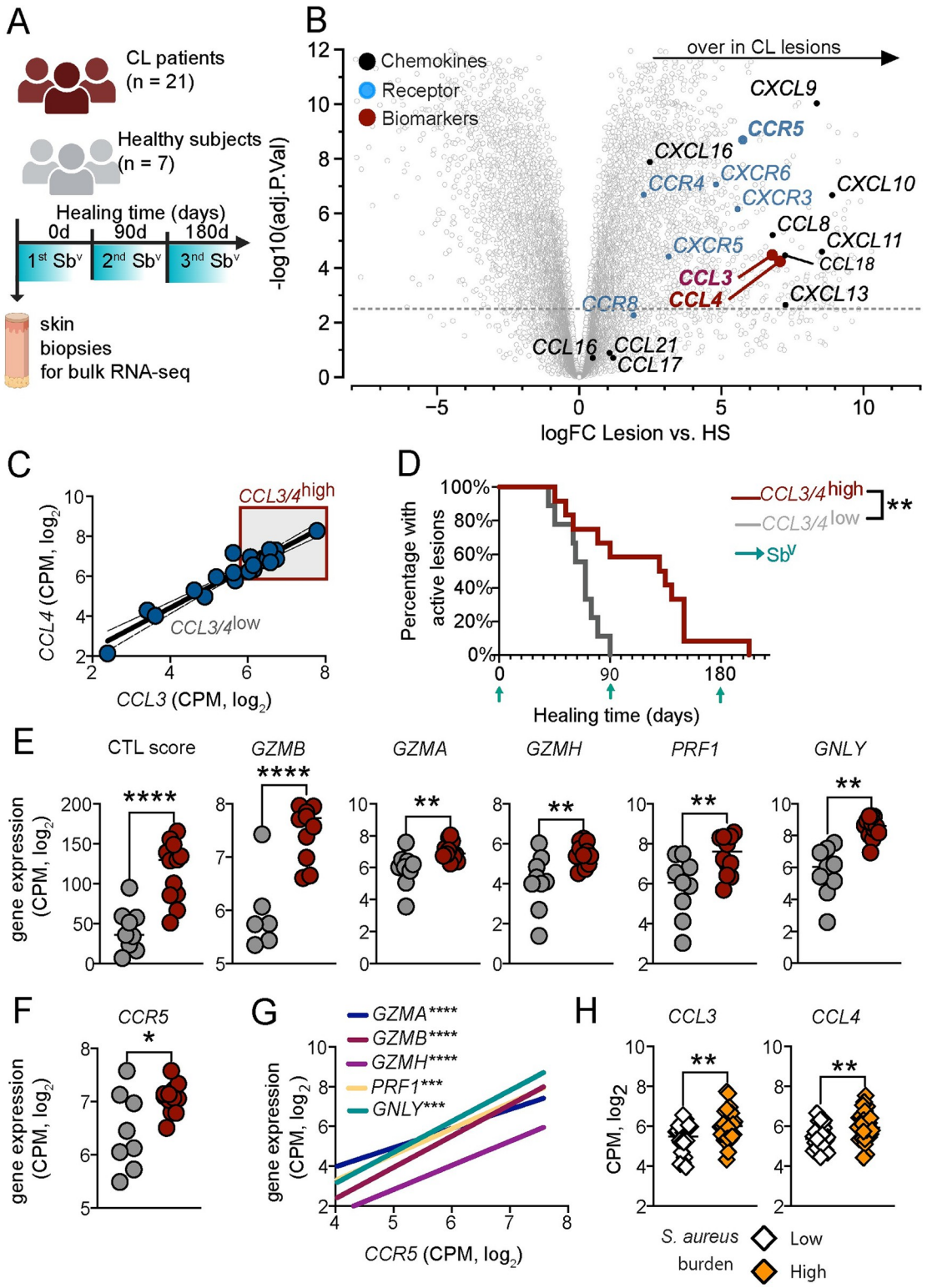

Fig 1. *CCR5* and its ligands are overexpressed in patient lesions and are associated with delayed healing. (A) The study design for the bulk RNA-seq dataset. Lesions were collected at day 0, and the complete re-epithelialization of lesions was followed at days 90 and 180 of antimony treatment. RNA-seq analysis of skin or lesions was performed with 7 healthy subjects (HS) and 21 cutaneous leishmaniasis patients (CL). (B) Volcano plot highlighting overexpressed chemokines (black), chemokines receptors (blue), and biomarkers that predict treatment outcome (red) in biopsies from patients relative to biopsies from healthy subjects.

(C) Correlation between *CCL4* and *CCL3* expression. *CCL3/4*^{high} expression was estimated based on *CCL3* > 5.8 and *CCL4* > 6. (D) Healing time of patients with high and low *CCL3/4* expression. (E) MCP counter abundance for cytotoxic T lymphocyte (CTL) score and *GZMB*, *GZMA*, *GZMH*, *PRF1*, and *GNLY* expression between patients with high and low expression of CCL3/4. (F) *CCR5* expression in patients with high and low *CCL3/4* expression. (G) Correlation between *CCR5* expression and *GZMB*, *GZMA*, *GZMH*, *PRF1*, and *GNLY* from CL patients. (H) *CCL3* and *CCL4* expression in patients with high and low *S. aureus* transcriptional abundance expression. Data was obtained from RNA-seq analysis of lesions from 51 patients. Gene expression is represented as counts per million (CPM) in the log2 scale. The clip art within the figure panel was made on Biorender. Statistical significance was determined using the two-tailed unpaired Student's t-test. Pearson correlation coefficient was used to determine the correlation between log2 expressions of genes from human skin. $^{*}p < 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p < .0001$.

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Since the increased pathology observed in *L. braziliensis* patients is mediated by the cytolytic activity of CD8^{+} T cells [8,20,21], we asked if CCL3/4^{high} expression was associated with CD8^{+} T cells. Using MCP-counter as a method to estimate cell abundances from unstructured RNAseq data [22], we found an association between CCL3/4^{high} expression and cytotoxic T lymphocyte abundances (CTL score) (Fig 1E). Among genes associated with cytolysis, *GZMB*, *GZMA*, *GZMH*, *PRF1*, and *GNLY* are the most differentially expressed in the patients' lesions and peripheral blood [7,23]. Given that cytotoxicity-related genes are associated with treatment outcome [7], we further evaluated whether the genes encoding for the cytolytic machinery were associated with CCL3/4^{high} expression. We observed an overexpression of *GZMB*, *GZMA*, *GZMH*, *PRF1*, and *GNLY* in patients with CCL3/4^{high} expression compared to CCL3/4^{low} expression (Fig 1E). Additionally, patients with CCL3/4^{high} expression exhibited increased *CCR5* expression, suggesting that the high abundance of these chemokines leads to the recruitment of CCR5^{+} cells (Fig 1F). Furthermore, we found a significant positive correlation between *CCR5* expression and *GZMB*, *GZMA*, *GZMH*, *PRF1*, and *GNLY* at lesions (Fig 1G). The correlation between *CCR5* expression and cytolytic granules in healthy skin samples was not notably strong. While a few parametric tests showed p-values slightly exceeding thresholds, this pattern does not parallel the observed results in patient samples (S2 Fig). This reinforces the evidence of a correlated network expression between CCR5 and transcriptional cytotoxic activity in *L. braziliensis*-patients.

Investigating whether *CCR5* expression correlates with the estimated cell abundances for other hematopoietic cells, we found a positive correlation between *CCR5* expression and total T cells ($p < 0.0001$), monocytes/macrophages ($p < 0.001$) and no correlation with neutrophils and dendritic cells (S3 Fig). Since the high abundance of *S. aureus* in *L. braziliensis* lesions is associated with increased expression of cytolytic genes and delayed healing [24], we next evaluated if there is an association between the expression of *CCL3* and *CCL4* with a high or low transcriptional abundance of *S. aureus*. We found that patients with a high abundance of *S. aureus* have an overexpression of *CCL3* and *CCL4* (Fig 1H). Together, these data demonstrate that CCR5 and its ligands are enriched in patients' lesions and are associated with a delayed healing time of lesions.

CCL3 and CCL4 are enriched systemically in patients and are associated with cytolytic gene expression

Despite being a localized skin infection, the systemic transcriptional signatures of *L. braziliensis* patients reflect pathways that are present in leishmanial lesions [23]. To address whether *CCR5* and its ligands were overexpressed at the systemic level, we analyzed an RNA-seq dataset from the peripheral blood of 50 cutaneous leishmaniasis patients and 14 healthy subjects (Fig 2A). We observed that *CCL3* and *CCL4* were elevated in the peripheral blood of *L. braziliensis* patients relative to healthy subjects (Fig 2B). Additionally, there was a positive correlation between the expression of *CCL3*, *CCL4*, and *CCR5* with the expression of cytolytic genes

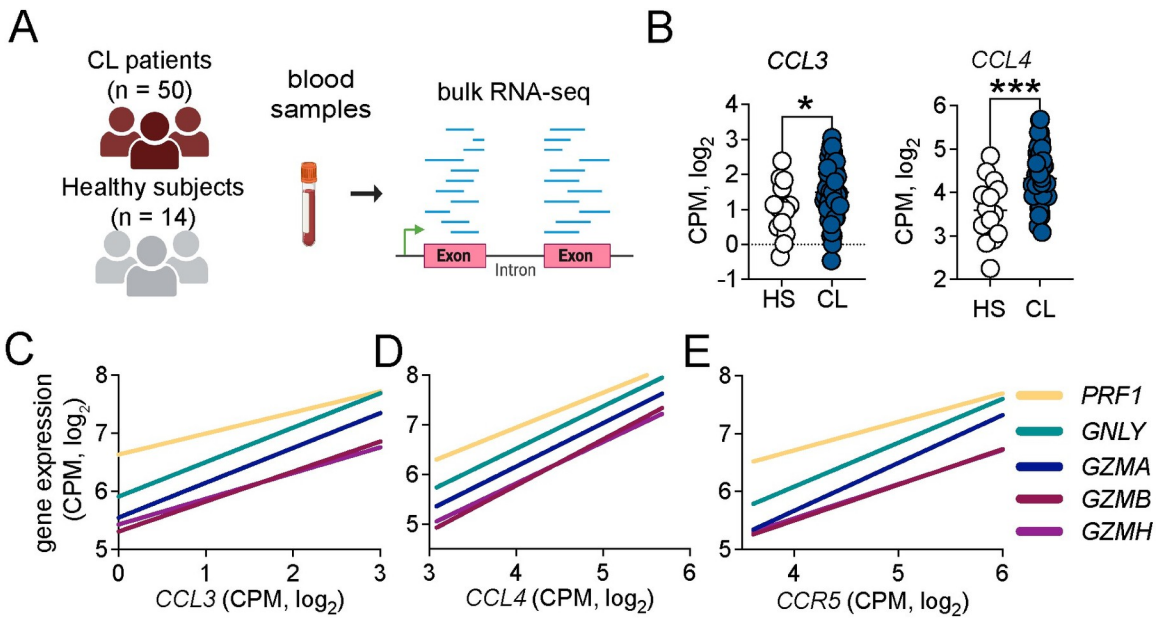

Fig 2. Systemic expression of CCR5 ligands is associated with cytolytic molecules. (A) RNA-seq analysis of peripheral blood from 14 healthy subjects (HS) compared to 50 cutaneous leishmaniasis patients (CL). (B) Gene expression of *CCL3* and *CCL4*. (C-E) Correlation of *GZMA*, *GZMB*, *GZMH*, *GLNY*, and *PRF1* with (C) *CCL3*, (D) *CCL4*, and (E) *CCR5* from CL patients. Gene expression is represented as counts per million (CPM) in the log2 scale. The clip art within the figure panel was made on Biorender. Statistical significance was determined using the two-tailed unpaired Student's t-test. Pearson correlation coefficient was used to determine the correlation between log2 expressions of genes from peripheral blood. $^{*}p < 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p < .0001$.

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GZMB, *GZMA*, *GZMH*, *GNLY*, and *PRF1* (Fig 2C–2E). These data suggest that the systemic transcriptional signature for CCR5 and its ligands observed in cutaneous leishmaniasis patients recapitulates the signature in lesions. Together, combining the transcriptional data from lesions and peripheral blood, our results suggest that CCR5 is associated with $CD8^+$ T cells cytolytic machinery components during human cutaneous leishmaniasis.

IL-15 upregulates CCR5 expression on $CD8^+$ T cells from patients

IL15 signaling is elevated in *L. braziliensis* lesions [25], and its inhibition lessens $CD8^+$ T cell-mediated pathology [6]. Given the role of IL-15 driving $CD8^+$ T cell migration by upregulating CCR5 expression [13], we focused our attention on exploring whether IL-15 contributes to the upregulation of CCR5 on $CD8^+$ T cells. We first evaluated *IL15* expression by analyzing the same RNA-seq dataset from the peripheral blood used in Fig 2 [23]. We observed that *IL15* is enriched systemically in *L. braziliensis* patients compared to healthy subjects (Fig 3A). Additionally, there was a significant positive correlation between the expression of *IL15* and *CCR5* in the peripheral blood of patients (r = 0.32, p = 0.02) (Fig 3B). No correlation was found in healthy subjects (r = 0.31, p = 0.27) (Fig 3C). These data suggest that the increase in *CCR5* expression is likely because of increased *IL15* expression in the peripheral blood of patients. To address the direct effect of IL-15 in the upregulation of CCR5 on $CD8^+$ T cells from *L. braziliensis* patients, we obtained peripheral blood mononuclear cells (PBMCs) from patients or healthy subjects and stimulated the cells with recombinant IL-15. After 18h, CCR5 expression by $CD8^+$ T cells was evaluated by flow cytometry. We observed that IL-15 stimulation enhances the frequency of $CD8^+$ T cells expressing CCR5 and the median fluorescence intensity (MFI) from patients (Fig 3D). In contrast, the baseline expression of CCR5 was lower in healthy subjects, and the increase induced by IL-15 was variable (Fig 3E). Collectively, the

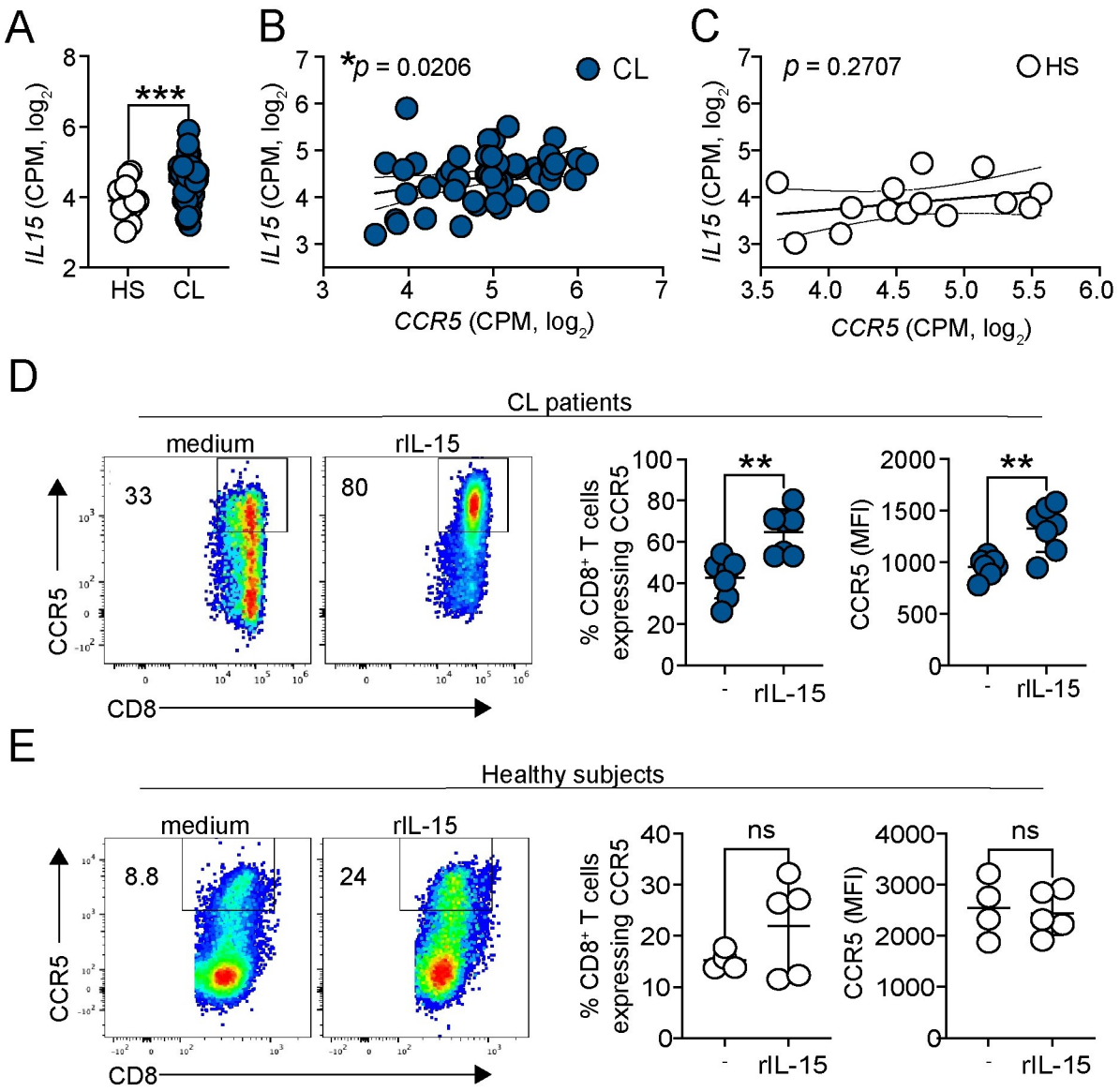

Fig 3. IL-15 upregulates CCR5 expression on CD8$^+$ T cells from patients. RNASeq analysis of peripheral blood from 14 healthy subjects (HS) compared to 50 cutaneous leishmaniasis patients (CL). (A) Gene expression of *IL15*. (B and C) Correlation between *IL15* and *CCR5* expressions of (B) CL and (C) HS. Gene expression is represented as counts per million (CPM) in the log2 scale. (D and E) PBMCs from (D) cutaneous leishmaniasis patients and (E) healthy subjects were cultured with recombinant IL-15 for 18h and stained for flow cytometry. Dot plots and graph bars represent the percentage and median fluorescence intensity (MFI) of CCR5 expression by CD8$^+$ T cells after IL-15 stimulation. Data were obtained from 5 HS and 7 cutaneous leishmaniasis patients. CL, cutaneous leishmaniasis; HS, healthy subjects; PBMC, peripheral blood mononuclear cells. Statistical significance was determined using the two-tailed unpaired Student's t-test. Pearson correlation coefficient was used to determine the correlation between log2 expressions of genes from peripheral blood. $^*p < 0.05$, $^{**}p \leq 0.01$.

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results demonstrate that IL-15 upregulates CCR5 expression on CD8$^+$ T cells from *L. braziliensis* patients.

CD8$^+$ T cells express CCR5 in leishmanial lesions in mice

The results of transcriptional analysis of lesions and peripheral blood from patients suggest that CCR5 promotes CD8$^+$ T cell migration to lesions. To determine if CCR5 was associated with more severe disease in mice, we evaluated *Ccl3*, *Ccl4*, and *Ccr5* gene expression in lesions

from two murine models of severe disease in murine cutaneous leishmaniasis (Fig 4A and 4C). Several studies have shown that in cutaneous leishmaniasis colonization of the skin with *Staphylococcus spp*. promotes increased pathology in mice or treatment failure in patients [26–29]. Therefore, we colonized mice with *S. epidermidis* and infected with *L. major* to assess if *Ccl3*, *Ccl4* and *Ccr5* expression was increased in mice with more severe disease. Mice colonized with *S. epidermidis* developed larger lesions than control mice [30]. Transcriptional analysis of cells from infected ears demonstrated that *Ccl3*, *Ccl4*, and *Ccr5* were significantly induced in both *S. epi*-colonized and *L. major*-infected mice, compared to mice only *S. epi*-colonized (*Ccl3*, $P < 0.0001$; *Ccl4*, $P < 0.0001$; *Ccr5*, $P < 0.0001$) as well as in *L. major*-infected mice alone (*Ccl3*, $P < 0.0001$; *Ccl4*, $P = 0.0002$; *Ccr5*, $P = 0.01$) (Fig 4B). These results suggest that CCL3, CCL4 and CCR5 likely not only contribute to CD8 T cell recruitment in *Staphylococcus* colonized *L. major* infected mice, but also in mice only infected with *L. major*.

We also examined *Ccl3*, *Ccl4*, and *Ccr5* expression in an experimental model that mimics the pathologic profile of cytotoxic CD8$^+$ T cells observed in human lesions [7,8]. In this model, the reconstitution of Rag1$^{-/-}$ mice with CD8$^+$ T cells alone leads to the development of uncontrolled lesions in a perforin-dependent manner [8]. CD8$^+$ T cells transferred in the absence of CD4$^+$ T cells are more pathological than CD8$^+$ T cells co-transferred with CD4$^+$ T cells [8] because CD4$^+$ Th1 cells and CD4$^+$ T regulatory dampen the immune response during leishmaniasis [31,32]. The severe pathology is dependent on the CD8$^+$ T cell cytolytic function of perforin and granzyme B [8] as well as the production of IL-1β [10], similar to what is described in patients' lesions and is associated with a large increase of neutrophils [7,8]. Transcriptional analysis of infected ears demonstrated that *Ccl3*, *Ccl4*, and *Ccr5* were significantly induced in *L. braziliensis*-infected Rag1$^{-/-}$ lesions that received only CD8$^+$ T cells, compared to mice that received both CD4+CD8 cells (*Ccl3*, $P = 0.0001$; *Ccl4*, $P = 0.0001$; *Ccr5*, $P = 0.05$) or mice that did not receive T cells (*Ccl3*, $P < 0.0001$; *Ccl4*, $P = 0.0002$; *Ccr5*, $P = 0.009$) (Fig 4D). CD8$^+$ T cells from *L. braziliensis*-infected Rag1$^{-/-}$ mice were also analyzed by flow cytometry, and 10% of CD8$^+$ T cells from infected ears expressed CCR5, while less than 1% of CD8$^+$ T cells obtained from dLN and spleen expressed CCR5 (Fig 4E). Thus, CD8$^+$ T cells express CCR5 preferentially at the lesions compared to the dLN. As myeloid cells also express CCR5 [33,34], we analyzed the CCR5 expression by CD11b$^+$ cells. We found that approximately 20% of CD11b$^+$ cells express CCR5 at the lesions and dLN (S4 Fig). Taken together, these results demonstrate that CCR5, as well as its ligands, is highly expressed in the murine models of severe human leishmaniasis.

CD8$^+$ T cell migration to the lesion is dependent on CCR5

To directly test if CCR5 expression on CD8$^+$ T cells was required to promote disease, *L. braziliensis*-infected Rag1$^{-/-}$ mice were reconstituted with WT or CCR5$^{-/-}$ CD8$^+$ T cells, and the course of infection was monitored (Fig 5A). As expected, Rag1$^{-/-}$ mice reconstituted with WT CD8$^+$ T cells (WT CD8) developed uncontrolled lesions. Importantly, Rag1$^{-/-}$ mice reconstituted with CCR5$^{-/-}$ CD8$^+$ T cells (CCR5$^{-/-}$ CD8) exhibited significantly smaller lesions (Fig 5B) with less pathology (Fig 5C and 5D). In contrast, parasite burdens were similar in Rag1$^{-/-}$ mice that received WT or CCR5$^{-/-}$ CD8$^+$ T cells (Fig 5E). Consistent with this reduced pathology, we observed a significant reduction in the frequency and number of CD8$^+$ T cells in the lesion of Rag1$^{-/-}$ mice that received CCR5$^{-/-}$ CD8$^+$ T cells compared to Rag1$^{-/-}$ mice that received WT CD8$^+$ T cells (Fig 5F–5H).

Neutrophils are persistently recruited to the lesions as a consequence of the cytolytic activity of CD8$^+$ T cells and are the main source of IL-1β during chronic infection [7,8,10]. We observed that Rag1$^{-/-}$ + CCR5$^{-/-}$ CD8 mice had a significant reduction in the frequency and

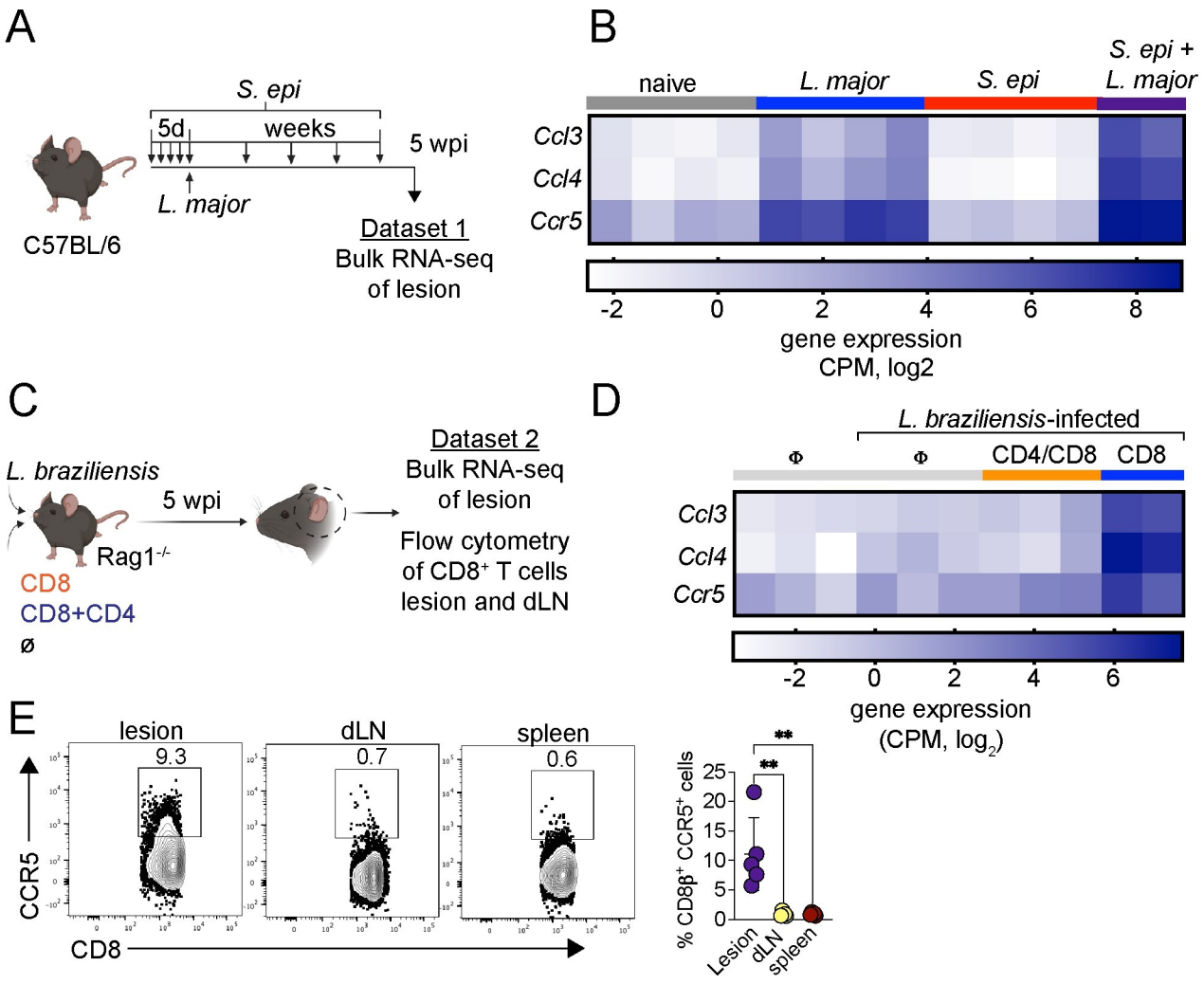

Fig 4. CD8$^+$ T cells express CCR5 in leishmanial lesions. (A) C57BL/6 mice were topically colonized with 10^8 *S. epidermidis* (*S. epi*) every day for a total of five applications and then intradermally infected with 2 x 10^6 *L. major* parasites. Mice were colonized once per week for the duration of the experiment. At 5 weeks post-infection, lesions were obtained for bulk RNA-seq analysis. (B) Heat map showing gene expression of *Ccl3*, *Ccl4*, and *Ccr5* in the lesions of naïve C57BL/6 mice, *L. major*-infected, *S. epidermidis* -colonized or *S. epidermidis* -colonized and *L. major*-infected mice (*S. epidermidis* + *L. major*). (C) Rag1$^{-/-}$ mice were infected with *L. braziliensis* in the ear and reconstituted with either CD8$^+$ T cells (CD8) or CD8$^+$ and CD4$^+$ T cells (CD8+CD4) or did not receive cells (∅). At the peak of infection, lesions were obtained for bulk RNA-seq analysis. (D) Heat map showing gene expression of *Ccl3*, *Ccl4*, and *Ccr5* in the lesions of naïve Rag1$^{-/-}$ mice and infected-Rag1$^{-/-}$ mice not reconstituted with cells and reconstituted with CD4$^+$/CD8$^+$ or only CD8$^+$ cells. Each column in the heatmap represents an individual mouse. Gene expression is represented as counts per million (CPM) in the log2 scale. (E) Representative dot plots and graph bars of CD8$^+$ T cells expressing CCR5 in the lesion, dLN and spleen of *L. braziliensis*-infected Rag1$^{-/-}$ reconstituted with CD8$^+$ T cells. The clip art within the figure panel was made on Biorender. The data (E) are expressed as the means ± SEMs and are representative of two independent experiments (n = 3–5 mice). Statistical significance was determined using two-tailed unpaired Student's t-test (E) or one-way ANOVA (B and D). $^{**}p < 0.01$ (E). *S. epi* + *L. major* vs. *S. epi* (*Ccl3*, P < 0.0001; *Ccl4*, P < 0.0001; *Ccr5*, P < 0.0001) or vs. *L. major* (*Ccl3*, P < 0.0001; *Ccl4*, P = 0.0002; *Ccr5*, P = 0.01) (B). CD8 vs. CD4+CD8 (*Ccl3*, P = 0.0001; *Ccl4*, P = 0.0001; *Ccr5*, P = 0.05) or vs. Φ (*Ccl3*, P < 0.0001; *Ccl4*, P = 0.0002; *Ccr5*, P = 0.009).

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number of neutrophils (CD11b$^+$ Ly6G$^+$ cells) (Fig 5I–5K) and in neutrophils expressing pro-IL-1β (Fig 5L–5N). Altogether, these results demonstrate that CD8$^+$ T cells migrate to the lesion in a CCR5-dependent manner.

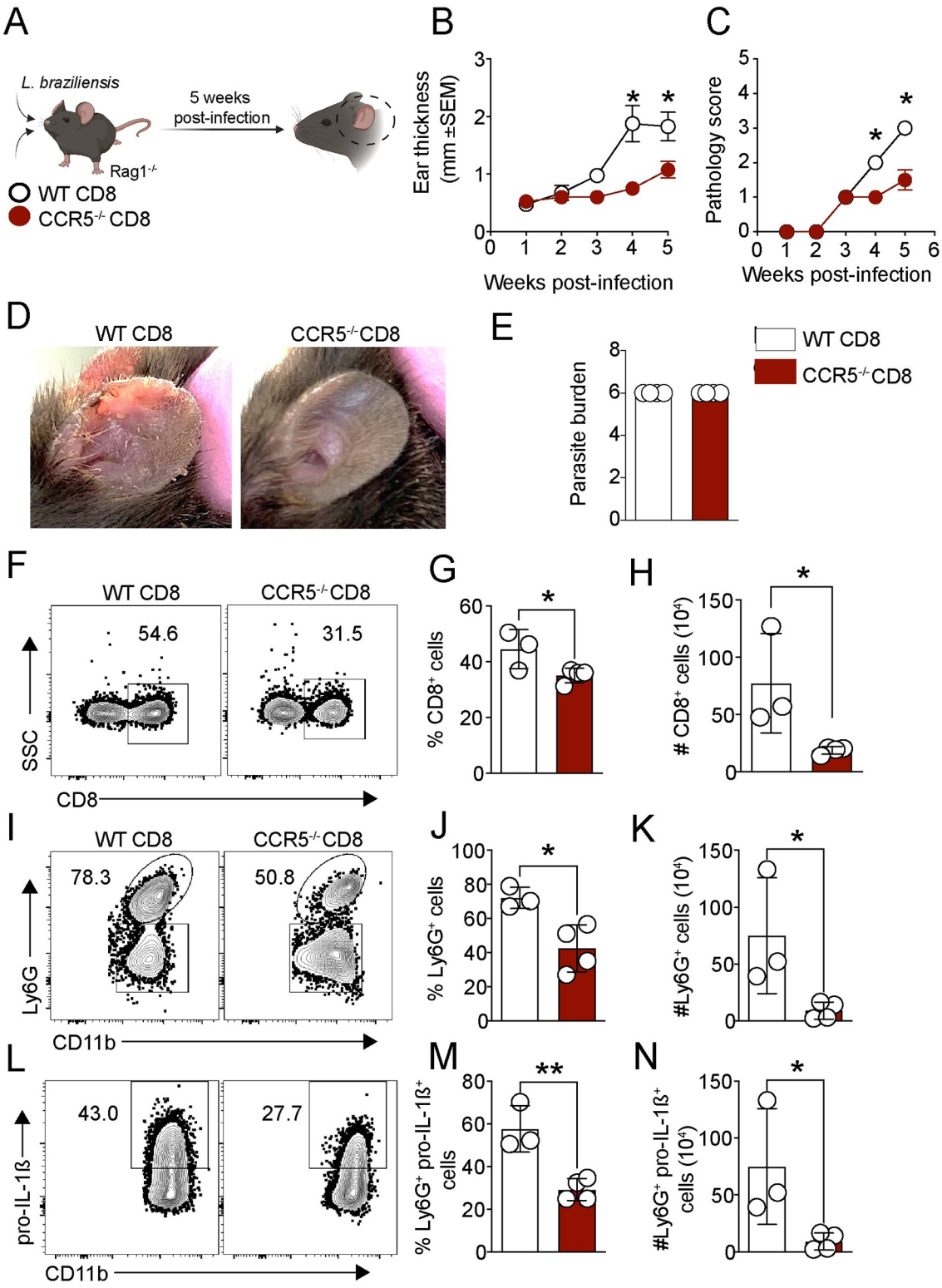

Fig 5. CD8+ T cell migration to the lesion is CCR5-dependent. (A) Rag1$^{-/-}$ mice were infected with *L. braziliensis* and reconstituted with WT or CCR5$^{-/-}$ CD8^{+} T cells. (B) Ear thickness and (C) pathology score was assessed weekly. (D) Representative pictures of lesions from infected-Rag1$^{-/-}$ that received WT CD8 or CCR5$^{-/-}$ CD8^{+} T cells were taken at 5 weeks post-infection. (E-N) At 5 weeks post-infection, mice were euthanized, and the ears were digested for (E) parasite quantification by limiting dilution and (F-N) flow cytometry analysis. (F) Representative dot plots and graph bars of the (G) frequency and (H) number of CD8^{+} T cells. (I-N) The

neutrophil number (CD11b$^+$ Ly6G$^+$ cells) and pro-IL-1β expression in neutrophils from the lesion were determined directly *ex vivo* by flow cytometry. (I-K) Representative dot plots and graph bars of neutrophils and (L-N) pro-IL-1β expression. The clip art within the figure panel was made on Biorender. The data are expressed as the means ± SEMs and are representative of two independent experiments (n = 3–5 mice/group). Statistical significance was determined using two-tailed unpaired Student's t-test. $^*p < 0.05$ *and* $^{**}p < 0.01$.

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CCR5 inhibition by maraviroc impairs CL pathology

To investigate the therapeutic potential of blocking CCR5, we took advantage of maraviroc (MVC), an FDA-approved drug used to treat HIV infection that selectively inhibits CCR5 [35]. We tested if MVC treatment abrogated CD8$^+$ T cell-mediated pathology. Infected-Rag1$^{-/-}$ mice reconstituted with CD8$^+$ T cells were treated daily with MVC by intraperitoneal injection or not treated as the control group (Fig 6A). As expected, control mice developed uncontrolled lesions with increased pathology, while mice treated with MVC showed a significant reduction in lesion size (Fig 6B) and minimum pathology (Fig 6C and 6D). No differences were observed in parasite numbers between MVC-treated and untreated mice (Fig 6E). We found that mice treated with MVC had a reduced frequency (Fig 6F and 6G) and number of CD8$^+$ T cells in the lesion (Fig 6H). Additionally, MVC-treated mice had a significantly reduced frequency and number of neutrophils (CD11b+ Ly6G$^+$ cells) (Fig 6I–6K) and a reduced number of neutrophils expressing pro-IL-1β (Fig 6L–6N). No differences were observed in macrophages, monocytes, and dendritic cell populations. Altogether, these data demonstrate that the inhibition of CCR5 prevents the CD8$^+$ cells mediated pathology without affecting parasite number.

Discussion

CD8$^+$ T cells mediate the destructive inflammation in *L. braziliensis* patients due to their cytolytic activity leading to cell death, NLRP3 activation, IL-1β secretion, and consequently exacerbated inflammation [7–9]. We previously demonstrated that blocking this pathway ameliorates the severity of the disease in murine models [10]. Here, we investigated the factors involved in CD8$^+$ T cell migration to lesions in order to identify approaches to lessen disease severity. We identified CCR5 and its ligands, CCL3 and CCL4, as critical for CD8$^+$ T cell migration to the lesion. Importantly, we demonstrated that the treatment with maraviroc, a selective inhibitor of CCR5, significantly blocked CD8$^+$ T cell recruitment to leishmanial lesions and the development of severe disease without affecting parasite control.

Among the chemokine receptors related to T-cell migration analyzed in our study, we identified CCR5 as the most highly expressed in patients' lesions and found a correlation between CCR5 and cytolytic genes, which were previously described to be associated with treatment failure [7].

CCR5 is expressed by monocytes [36], dendritic cells [37], NK cells [38,39], and lymphocytes [40,41] and regulates the trafficking and effector function of those cells. CCR5 contributes to the pathogenesis of numerous diseases, including graft-versus-host disease [42], autoimmune diseases [43,44], and infectious diseases [13,45]. CCR5 also promotes the migration of memory and effector-specific CD8$^+$ T cells to peripheral tissues and plays a beneficial role in controlling viral and toxoplasma replication in the lungs [46] and intestine [47], respectively. However, consistent with our results, CCR5 is deleterious in situations where the cytolytic activity of CD8$^+$ T cells leads to tissue damage, for example, in cerebral malaria [45], *T. cruzi*-elicited cardiomyopathy [48], acute hepatitis caused by HAV infection [13], and alopecia areata [49]. In these situations, CCR5 inhibition or deletion limits CD8$^+$ T cell-mediated pathology.

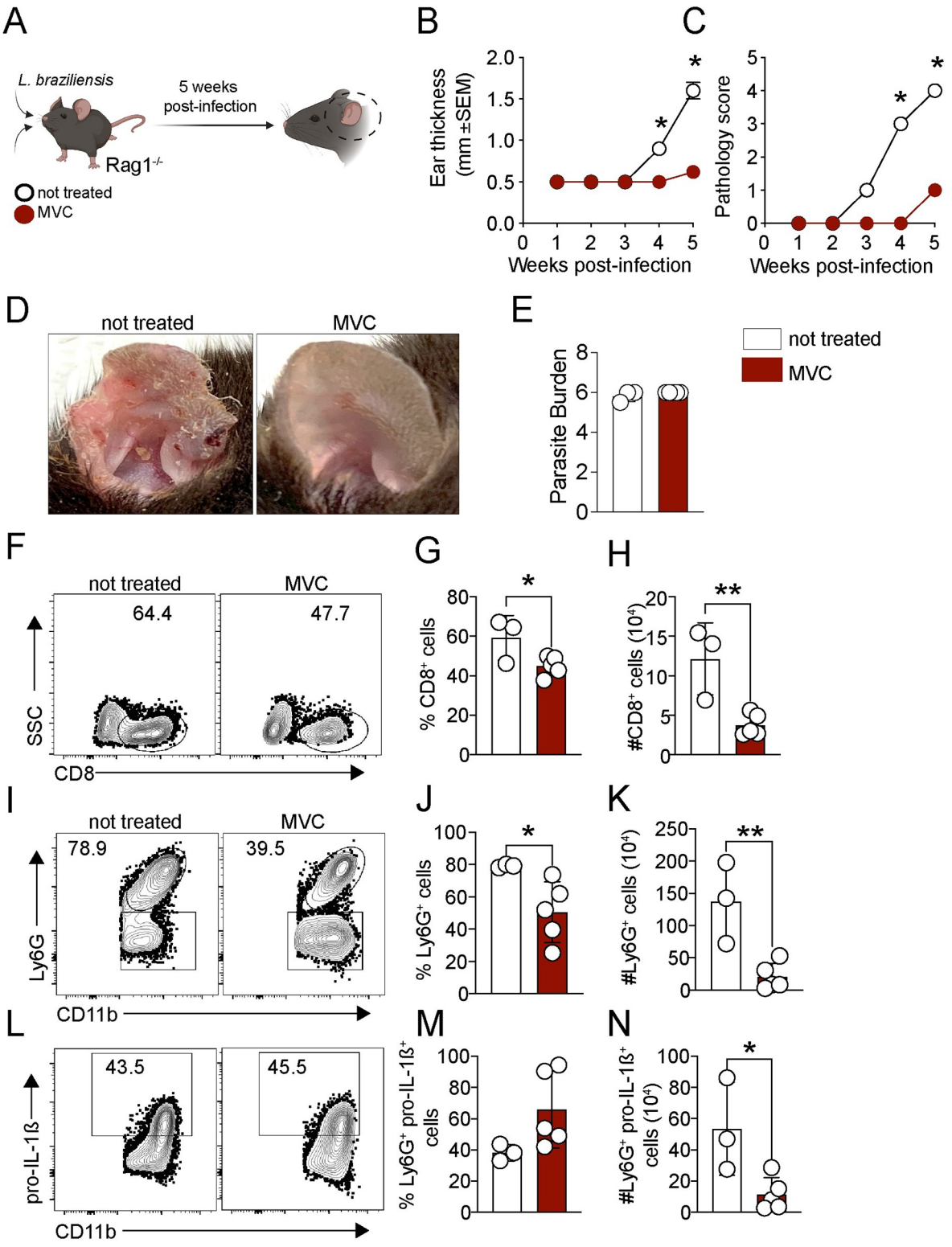

Fig 6. CCR5 inhibition prevents CD8+ T cell-dependent immunopathology. (A) Rag1$^{-/-}$ mice were infected with *L. braziliensis*, reconstituted with CD8+ T cells and treated daily with maraviroc (MVC). (B) Ear thickness and (C) pathology score was evaluated weekly. (D) Representative pictures of lesions from infected-Rag1$^{-/-}$ treated with MVC or not treated were taken at 5 weeks post-infection. (E-N) At 5 weeks post-infection, mice were euthanized, and the ears were digested for (E) parasite quantification by limiting dilution and (F-N) flow cytometry analysis. (F) Representative dot plots and bar graphs of the (G) frequency and (H) number of CD8+ T cells. (I-N) The neutrophil

number (CD11b$^+$ Ly6G$^+$ cells) and pro-IL-1β expression in neutrophils from the lesion were determined directly *ex vivo* by flow cytometry. (I-K) Representative dot plots and graph bars of neutrophils and (L-N) pro-IL-1β expression. The clip art within the figure panel was made on Biorender. The data are expressed as the means ± SEMs and are representative of two independent experiments (n = 3–5 mice). Statistical significance was determined using two-tailed unpaired Student's t-test. $^*p < 0.05$ *and* $^{**}p < 0.01$.

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The biological effect of CCR5 is mediated by its interaction with the chemokines CCL3 and CCL4 [50,51]. Our transcriptional analysis of lesions demonstrated that *L. braziliensis* patients can be classified by the high and low expression of *CCL3/4* at the lesions. Importantly, our data demonstrated that patients with *CCL3/4*^{high} expression had delayed healing of lesions and enrichment of cytolytic genes and *CCR5*. These results suggest that the increased expression of *CCL3* and *CCL4* leads to the recruitment of cytolytic CD8$^+$ T cells. CCL3 and CCL4 are produced by dendritic cells [52], neutrophils [53], lymphocytes [54], and non-hematopoietic cells, such as endothelial and epithelial cells [55] in the peripheral tissue in situations of inflammation or infection, and orchestrate the immune responses by promoting the recruitment of CCR5-expressing leukocytes and also by contributing to their effector functions [13,53,56]. Additionally, in silico tumor simulations demonstrated that CCL3 and CCL4 attract CTLs into the tumor, and the newly arriving CTLs amplify chemokine production and promote a positive feedback loop of recruitment [57]. Thus, in addition to directly promoting pathology by cell death of infected cells, it is possible that recruited cytotoxic CD8$^+$ T cells at the *L. braziliensis* lesions may amplify and sustain the loop of inflammation that leads to increased pathology.

The most relevant gene expression in the patients' lesions is likely observed in the peripheral blood, such as cytotoxicity and (interferon-stimulated gene) ISG genes, for example. Consistently, serum levels of IFN-γ correlate with the peripheral ISG signature in CL patients [23]. Thus, the systemic transcriptional signatures reflect the most enriched gene signatures that are happening at the lesions and reinforce the findings. However, it is not possible to predict if the increased expression of *CCL3* and *CCL4* mRNAs in the peripheral blood of CL patients compared to HS captures the gene expression of circulating immune cells from the CL lesions or if circulating cells are activated by IFN-γ to enhance the expression of *CCL3* and *CCL4* mRNAs prior to lesion entry.

Interestingly, there is a polymorphism in CCR5 (CCR5Δ32 allele) that results in a non-functional CCR5, which is reported to prevent cell invasion by HIV-1 [58,59] and is implicated in defective cell chemotaxis [60]. A study investigating whether the CCR5 polymorphism influences the progression of cutaneous to mucocutaneous in a Brazilian cohort of *L. braziliensis*-infected patients found that no mucocutaneous patients were CCR5 polymorphism carriers, although due to the small sample size, no significant differences in the CCR5Δ32 frequency between cutaneous and mucocutaneous were found [61]. However, given the role we have described for CCR5, additional studies would be appropriate to evaluate if patients who rapidly cure *L. braziliensis* lesions are CCR5 polymorphism carriers versus patients who have a delay in healing.

To directly test the role of CCR5 expression in cytolytic CD8$^+$ T cells, we tested the role of CCR5 deficient CD8 T cells in promoting increased disease Rag1$^{-/-}$ mice. In this model, CD8$^+$ T cells promote pathology by a mechanism dependent on the cytolytic function of perforin and granzyme B, which leads to inflammatory infiltration and IL-1β release, similar to the phenotype we observe in patients' lesions [8,23]. An advantage of this model is that it allowed us to study the role of CCR5 specifically on CD8$^+$ T cells, which is important since many different cells express CCR5 [36,38,40,41]. We found that CD8$^+$ T cells express CCR5 preferentially at the lesions compared to the dLN. Consistent with our observation, CCR5 is mainly expressed by effector and memory CD8$^+$ T cells [13,45–47,49], while naïve CD8$^+$ T cells express CCR5 in

a transient manner in the draining lymph node [62,63]. Also, our results show that Rag1$^{-/-}$ mice reconstituted with CD8$^+$ T cells have an enrichment of *CCR5* and its ligands compared to Rag1$^{-/-}$ mice reconstituted with CD8$^+$ and CD4$^+$ T cells, suggesting that this chemotactic pathway is a feature of increased pathology derived from cytolytic CD8$^+$ T cells activity.

Although our results show that CCR5 has a critical role in driving CD8$^+$ T cell migration to the leishmanial lesions, we cannot exclude the participation of other chemokine receptors acting to promote CD8$^+$ T cell migration to the lesion. The transcriptional analysis identified enrichment of CXCR3 and its ligands CXCL9/10/11, in *L. braziliensis*-lesions compared to healthy skin. CXCR3 is required for memory CD8$^+$ T cell recruitment to the lung during *Mycobacterium tuberculosis* infection [64] and during intracellular parasitic infections [45,65]. For example, CXCR3 promotes the migration of CD8$^+$ T cells to the cardiac tissue and the brain in the context of *T. cruzi* and *Plasmodium infection*, respectively [45,65]. However, CXCR3 plays a beneficial role during cutaneous leishmaniasis by promoting the recruitment of Th1 cells to the lesions. Thus, *L. major*-infected CXCR3$^{-/-}$ mice are more susceptible to infection due to a reduced number of CD4$^+$T cells producing IFNγ in the lesion. Consequently, these mice fail to control parasite replication [66]. For these reasons, CXCR3 inhibition is not a good approach for host-directed therapy due to predictable adverse side effects.

The colonization of leishmanial lesions by a staphylococcal species is an important factor promoting increased disease [27–30]. The high abundance of *S. aureus* in patients' *L. braziliensis* lesions is associated with increased expression of cytolytic genes, CD8$^+$ T cells, and delayed healing [28]. We took advantage of the published human dataset and a murine model of increased pathology induced by the colonization of S. *epidermidis* to evaluate whether there is an association between the Staphylococcal species colonization and the expression of *CCL3* and *CCL4* in the leishmaniasis lesions. Corroborating the studies, we found that the high abundance of *S. aureus* in patients' lesions is associated with the overexpression of *CCL3* and *CCL4*, and *S. epi*-colonized and *Leishmania*-infected mice presented a high *ccl3* and *ccl4* expression at the lesion. The tissue damage mediated by CD8$^+$ T cells resulting from *Leishmania* infection may provide the environment for *S. aureus* to invade and replicate into the skin lesions. As a consequence, there is a release of mediators, such as IL-1β, which feeds the pro-inflammatory loop and promotes Immunopathology [28]. Thus, the inhibition of CD8$^+$ T cell migration by targeting CCR5 would interrupt the initial steps of the cascade that leads to immunopathology.

Investigating the factors involved in CD8$^+$ T cells migration to lesions, we found that IL-15 upregulates CCR5 expression on circulating CD8$^+$ T cells from *L. braziliensis*-infected patients. IL-15 promotes the activation, proliferation, and cytotoxicity of effector and memory CD8$^+$ T cells [67–69]. We previously found that IL-15 is enriched in *L. braziliensis* lesions, and inhibition of IL-15 signaling by tofacitinib ameliorates pathology in mice by dampening the cytotoxicity function of CD8$^+$ T cells [6]. In the current study, we found that *IL15* is enriched in patients in the blood, reinforcing the relevance of IL-15 expression in the disease. In agreement with our data, it has been reported that IL-15 is over-produced systemically during infections [67,70,71] and inflammatory diseases [72–74], and we hypothesize that the systemic expression of IL-15 may contribute to the activation and migration of circulating CD8$^+$ T cells. This idea is supported by the fact that IL-15-treated memory CD8$^+$ T cells migrate from the circulation to peripheral tissues in a CCR5-dependent manner [13]. We found a positive correlation between the systemic expression of *IL15* and *CCR5* and demonstrated that IL-15 upregulates CCR5 on circulating CD8$^+$ T cells. Additionally, the baseline expression of CCR5 by CD8$^+$ T cells from the peripheral blood was higher in CL patients, possibly because the circulating cells from the lesions have a preactivated state. We suggest that in cutaneous leishmaniasis, systemic IL-15 may contribute to the activation of circulating CD8$^+$ T cells and promote CCR5-dependent

migration to the site of infection. Concomitantly, CCR5 ligands are over-expressed in leishmanial lesions, which contributes to the recruitment of CCR5-expressing $CD8^+$ T cells to the site of infection. Once in the lesion, IL-15 can enhance the cytolytic activity of $CD8^+$ T cells and could also contribute to the retention of $CD8^+$ T cells by maintaining CCR5 expression.

There are important limitations to this study, one of which is the initiation of maraviroc treatment before the signals of lesion development, and this does not reflect what is practiced in clinical medicine. The patient often starts the treatment when the lesions are well-established, being the definitive diagnosis of leishmaniasis relies in part on the clinical manifestations of the disease. Based on the dynamic of sustained recruitment of $CD8^+$ T cells to the lesions, we predict that maraviroc treatment would dampen the subsequent $CD8^+$ T cell migration. Importantly, a subset of patients arrive at the clinic with what is considered early cutaneous leishmaniasis. They exhibit small non-ulcerated nodules that even with drug treatment often develop into full blown cutaneous lesions [5]. We predict that MVC treatment would particularly benefit these patients and lessen lesion development. Only a clinical trial blocking CCR5 would determine whether the lesions would be dampened by interrupting $CD8^+$ T cell migration. Another limitation is that we used the $Rag1^{-/-}$ model to test the hypothesis that CCR5 is important for $CD8^+$ T cell migration. The $Rag1^{-/-}$ model is an extreme but necessary model because once reconstituted with $CD8^+$ T cells, it is possible to mimic the increased lesions observed in human CL and the pathological profile played by the $CD8^+$ T cells. However, due to the absence of $CD4^+$ T cells, the infected $Rag^{-/-}$ mice reconstituted only with $CD8^+$ T cells do not control the parasite replication.

The current first-line treatment of cutaneous leishmaniasis in Brazil is pentavalent antimony, which has significant side effects and often requires multiple rounds of treatments [3,4,75]. Therefore, complementing drug treatment with host-directed therapy aimed at reducing pathologic immune responses would be beneficial. Our data demonstrated the efficacy of MVC treatment in blocking the pathology induced by $CD8^+$ T cells in a murine model, revealing a new possibility of effective host-directed therapy. Because MVC is an FDA–approved drug, our findings raise the possibility that it could be repurposed for treating a subset of patients with cutaneous leishmaniasis. MVC is a selective CCR5 antagonist and has been used successfully in many other situations. For example, MVC is a treatment for CCR5-tropic HIV infection [35,76,77]. MVC effectively protects against graft-versus-host disease by blocking the recruitment of alloreactive donor T-cell responses [12,78–80] and improves lesions in a murine model of alopecia areata by impairing $CD8^+$ T cell migration [49]. Thus, MVC could be readily tested in cutaneous leishmaniasis patients, given its oral route of administration and known safety profile. Additionally, it was reported that *L. major*-infected $CCR5^{-/-}$ mice developed smaller lesions and had reduced parasite number due to deficient recruitment of $CD4^+$ T regulatory cells to the lesion, leading to an enhanced Th1 response [81]. Similarly, *L. donovani*-infected $CCR5^{-/-}$ or $CCL3^{-/-}$ mice exhibited enhanced IFNγ antigen-specific in the chronic phase of the disease [82]. Therefore, we suggest that the inhibition of CCR5 might prevent the migration of pathogenic $CD8^+$ T cells without interfering with the protective immune mechanisms related to the control of parasite replication. Together, these findings identified an approach that can be employed as a treatment in combination with anti-parasitic drugs to ameliorate cutaneous leishmaniasis severity.

Material and methods

Ethics statement

This study was conducted according to the principles specified in the Declaration of Helsinki and under local ethical guidelines (Ethical Committee of the Maternidade Climerio de

Oliveira, Salvador, Bahia, Brazil; and the University of Pennsylvania Institutional Review Board). This study was approved by the Ethical Committee of the Federal University of Bahia (Salvador, Bahia, Brazil)(010/10) and the University of Pennsylvania IRB (Philadelphia, PA) (812026;823847). All patients provided written informed consent for the collection of samples and subsequent analysis. 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 Assurance Number 803457.

Patients

Seven cutaneous leishmaniasis patients were seen at the health post in Corte de Pedra, Bahia, Brazil. The criteria for diagnosis were a clinical characteristic of cutaneous leishmaniasis and parasite confirmation by PCR or positive delayed-type hypersensitivity response to *Leishmania* antigen. Exclusion criteria included previous anti-leishmanial treatment, individuals under 18 years old, pregnancy, or the presence of other comorbidities. Before treatment, 10 ml of peripheral blood was collected from CL patients and 5 endemic non-*L. braziliensis* infected controls (healthy subjects, HS). The blood samples for in vitro assays were collected in April 2022.

Mice

Six-to 8-week-old female mice were used for the infection experiments. C57BL/6 mice were purchased from Charles River, and CCR5$^{-/-}$ (B6.129P2-Ccr5tm1Kuz/J) and Rag1$^{-/-}$ (B6.129S7-Rag1tm1Mom/J) were purchased from the Jackson Laboratory. All mice were maintained in a specific pathogen–free facility with free access to food and water, nesting material, and housed at a temperature of 21˚C at the University of Pennsylvania Animal Care Facilities. All animals were used in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the guidelines of the University of Pennsylvania Institutional Animal Use and Care Committee. The protocol was approved by the Institutional Animal Care and Use Committee, University of Pennsylvania Animal Welfare Assurance.

Parasites and bacterial cultures

L. major parasites (strain WHO/MHOM/IL/80/Friedlin) and *L. braziliensis* parasites (strain MHOM/BR/01/BA788) were grown in Schneider's insect medium (GIBCO) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin per mL. Parasitic virulence was maintained by serial passaging in BALB/c mice and by culturing *in vitro* for no more than five passages. An isolate of *Staphylococcus epidermidis* was cultured from the ears of *L. major* infected mice [83]. For topical associations and infections, the bacteria were cultured in Brain heart infusion (BHI) media (Remel, Lenexa, KS, USA) shaking for 12 hr at 37˚C.

Peripheral blood mononuclear cell cultures

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation using a Ficoll-Paque Plus gradient (GE Healthcare, Cat #17-1440-02), then washed by centrifugation, and resuspended in RPMI 1640 media (Gibco) supplemented with 10% FBS (Gibco) and 100 U/ml penicillin, and 100 μ g/ml streptomycin (Gibco). The PBMCs were adjusted to a concentration of 1×10^6 cells/mL in 500 mL of RPMI 1640 media. Culturing was performed in the presence

or absence of recombinant IL-15 (10 ng/mL) (Petrotech, Cat. #200-01B) and incubated for 18h at 37˚C under 5% CO_2 for 18h.

Human cutaneous leishmaniasis transcriptional profiling by RNA-seq

The analyses carried out in this study with the lesion and blood human datasets Amorim et al. 2019, Amorim et al. 2023, and Amorim et al. 2021 [7,23,24] were performed from the filtered, normalized gene expression matrix available for download as a text file on NCBI GEO accession GSE127831 and GSE162760. The list of six chemokine receptors associated with T-cell migration was collected based on literature [12–19]. Differential gene expression analysis was performed with edgeR R package [84]. Volcano plot was performed using DataGraph (Visual Data Tools). MCP-counter [22] and immundeconv R packages [85] were combined to estimate cytotoxic T cell abundances (CTL score) from the unstructured lesional RNA-seq dataset. *S. aureus* transcript identification, quantification, and patient stratification between *S. aureus* high vs. low were described [24]. Briefly, the dual RNA-seq computational approach was performed, in which the reads were mapped to both the human transcriptome and *S. aureus* pangenome (NCBI GEO accession GSE214397) to estimate total bacterial transcript abundances.

Intradermal infections and bacterial topical association

Infective-stage promastigotes (metacyclics) were isolated from 4–5-day old stationary culture by density gradient separation by Ficoll (Sigma-Aldrich, Cat #F9378). Mice were infected with 10^5 *L. braziliensis* metacyclic enriched promastigotes intradermally in the left ear. Lesion development was evaluated weekly by ear thickness with a digital caliper (Fisher Scientific), and the pathology was scored from 0 to 5 based on the criteria: 0) absence of lesion; 1) swelling/redness; 2) deformation of ear pinna; 3) ulceration; 4) partial tissue loss; 5) total tissue loss. Representative photos of lesions were taken at 5 weeks post-infection (Figs 5 and 6). For topical associations,10^8 CFUs of *Staphylococcus epidermidis* were applied to the entire mouse body using sterile cotton swabs every other day for a total of five times.

Cell purification and adoptive transfer

Spleen cells from C57BL/6 mice were collected, erythrocytes lysed with ACK lysing buffer (Quality Biological, Cat #118-156-101), and CD8$^+$ T cells were purified using a magnetic bead separation kit (Miltenyi Biotec, Cat #130-104-075). Rag1$^{-/-}$ mice were reconstituted with CD8$^+$ T cells by intravenous route (3 x 10^6 cells/mouse) and subsequently were infected with *L. braziliensis*. Mice received 250 mg of antibody anti-CD4, clone GK1.5 (BioXCell, Cat #BE0003-1) by intraperitoneal injections twice a week in the first two weeks post-infection [8].

Ear preparation and parasite titration

Infected ears were collected, the dorsal and ventral layers of the ear were split mechanically and placed dermis side down in a 24 wells plate with 500 μl/well of RPMI with 250 mg/mL of Libarase (Roche, Cat #05401054001) and 10 mg/mL of DNase I (Sigma-Aldrich, Cat #4536282001) for 90 min at 37˚C, 5% CO_2. The enzyme reaction was stopped with 1 mL of RPMI supplemented with 10% FBS. Ears were dissociated using a cell strainer (40mm) in PBS containing 0.05% BSA and 20 mM EDTA (Invitrogen, Cat #130-104-075), and the cell suspension was used for parasite titration. 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 days.

Mouse cutaneous leishmaniasis transcriptional profiling by RNA-seq

Two RNA-seq datasets were processed for this study: Dataset 1) C57BL/6 mice were topically colonized with 10^8 *S. epidermidis* every day for a total of five applications and then intradermally infected with 2 x 10^6 *L. major* parasites. Mice were colonized once per week for the duration of the experiment. At 5 weeks post-infection, lesions were obtained for bulk RNA-seq. Dataset 2) Rag1$^{-/-}$ mice were infected with *L. braziliensis* and subsequently reconstituted with CD8$^+$ T cells alone or CD8$^+$ and CD4$^+$ T cells or did not receive any T cells (ϕ). 5 weeks post-infection, mice were euthanized, and ears were collected in RNAlater (ThermoFisher, Cat #AM7020) for bulk RNA-seq studies. For both datasets, RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN, Cat #74106) according to the manufacturer's instructions and used to prepare Poly(A)+-enriched cDNA libraries Illumina TruSeq Stranded mRNA library prep workflow. Ribo-Zero Gold rRNA depletion (Illumina, Cat # MRZG12324) was used to remove ribosomal content. Quality assessment and quantification of RNA preparations and libraries were carried out using an Agilent 4200 TapeStation and Qubit 3, respectively. Samples were sequenced on an Illumina NextSeq 500 The raw reads were mapped to the mouse reference transcriptome (Ensembl; Mus musculus) using Kallisto [86]. All subsequent analyses were conducted using the statistical computing environment R (version 4.1.0), RStudio (version 1.4.1717), and Bioconductor (version 3.13). Transcript quantification, normalization, filtering for highly expressed genes, variance-stabilization, and Differential Gene Expression analysis were performed as described previously [24]. Raw sequence data are available at the NCBI GEO BioProjects GSE245292 and GSE260729.

Flow cytometry

Single-cell suspensions were stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Molecular Probes, Cat #L34957) and subsequently incubated with anti-CD16/CD32 (eBioscience, Cat #14-0161-86, clone 93) and 10% rat-IgG1 (Sigma-Aldrich, Cat #I8015) in PBS containing 0.1% BSA (Sigma-Aldrich). For surface staining, cells were incubated with monoclonal antibodies anti-mouse (anti-CD45 APCcy7 [clone30-F11, Cat #103116], anti-CD3 BV605 [clone 172A, Biolegend, Cat #100237], anti-CD8 BV711 [clone 53–6.7, Biolegend, Cat #100759], anti-Ly6G pacific blue [clone 1A8, Biolegend, Cat #127612], anti-F4/80 BV605 [clone BM8, Biolegend, Cat #123133], anti-CD11c BV711 [clone N418, Biolegend, Cat #117349], anti-Ly6C BV785 [clone HK1.4, Biolegend, Cat #128041], anti-MHCII Alexa fluor-700 [clone M5/114.15.2, eBiosciences, Cat #56-5321-82], anti-CD44 eF450 [clone IM7, Biosciences, Cat #48-0441-82], and anti-CCR5 PE [clone C34-3448, eBiosciences, Cat #559923] or anti-human antibodies (anti-CD45 Pecy7 [clone HI30, eBiosciences, Cat #25-0459-42], anti-CD8 Percp-cy5.5 [clone SK1, Biolegend, Cat #344710], anti-TCRa/b APC [clone IP26, eBiosciences, Cat #17-9986-42] and anti-CCR5 BV421 [clone 2D7, BD Biosciences, Cat #562576]), followed by fixation with 2% of formaldehyde and permeablization with 0.2% saponin/PBS. For intracellular staining, cells obtained from mice were permeabilized with 0.4% saponin buffer and stained *ex vivo* for pro-IL-1b APC (clone NJTEN3, eBiosciences, Cat #17-7114-80). Data were collected using LSRIII Fortessa (BD Biosciences) and analyzed using FlowJo software v10.6.2. (Tree Star).

Maraviroc treatment

Mice were intraperitoneally treated with 20 mg/kg of Maraviroc (Cayman Chemical, Cat #14641, CAS Number 376348-65-1) diluted in 10% DMSO (Sigma-Aldrich, Cat # D8418, CAS number 67-68-5) (200 ml/mouse) daily. The treatment started on the same day of infection. As a control, mice were treated intraperitoneally with 10% DMSO.

Quantification and statistical analysis

Data are shown as means ± SEM. For mouse and human experiments, statistical significance was determined using the two-tailed unpaired Student's t-test or one-way ANOVA. Pearson correlation coefficient was used to determine the correlation between log2 expressions of genes from human skin and peripheral blood transcripts. All statistical analysis was calculated using GraphPad Prism version 10 (GraphPad Software). Differences were considered significant when $^*p < 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p < .0001$. For human and mouse studies, specific sample sizes are represented by n and are indicated in figure legends.

Supporting information

S1 Fig. *CCL3* and *CCL4* expression at the lesions of patients that cured versus failed the pentavalent antimony treatment. At day 90 after the start of treatment, patients with complete re-epithelialization of lesions and the resolution of inflamed borders were considered cured, or patients with active lesions at 90 days were defined as failing, <90d or >90d, respectively. Data was obtained from RNA-Seq analysis of lesions from 21 patients and 7 healthy skin. Gene expression is represented as counts per million (CPM) in the log2 scale. Statistical significance was determined using two-tailed unpaired Student's t-test. $^*p < 0.05$.
(DOCX)

S2 Fig. Correlations between *CCR5* expression and cytolytic molecules in CL patients and healthy skin. Correlation between *CCR5* expression and *GZMB*, *GZMA*, *GZMH*, *PRF1*, and *GNLY* expression between CL patients (A) and healthy skin (B). Data was obtained from RNA-Seq analysis of lesions from 21 patients and 7 healthy skin. Gene expression is represented as counts per million (CPM) in the log2 scale. Pearson correlation coefficient was used to determine the correlation between log2 expressions of *CCR5* from human skin. $^*p < 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p < .0001$.
(DOCX)

S3 Fig. Correlations between CCR5 expression and estimated cell abundances at the *L. braziliensis*-lesions. Correlation between *CCR5* expression and MCP counter abundance for T cells, monocytes/macrophages, neutrophils, and mDC at the *L. braziliensis*-lesion. Data was obtained from RNA-seq analysis of lesions from 21 patients. Gene expression is represented as counts per million (CPM) in the log2 scale. Pearson correlation coefficient was used to determine the correlation between log2 expressions of *CCR5* from human skin. $^*p < 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p < .0001$.
(DOCX)

S4 Fig. CCR5 expression by CD11b$^+$ cells in leishmanial lesions. Representative dot plots and graph bars of CD11b$^+$ cells expressing CCR5 in the lesion and dLN of *L. braziliensis*-infected Rag1$^{-/-}$ reconstituted with CD8$^+$ T cells at 5 weeks post-infection. Statistical significance was determined using two-tailed unpaired Student's t-test.
(DOCX)

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