Transgenic expression of CXCR3 on T cells enhances susceptibility to cutaneous *Leishmania major* infection by inhibiting monocyte maturation and promoting a Th2 response

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Running Title: Transgenic CXCR3 expression enhances CL susceptibility.

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Abstract:

Cutaneous leishmaniasis, caused mainly by *Leishmania major*, an obligate intracellular parasite, is a disfiguring disease characterized by large skin lesions and is transmitted by a sand-fly vector. We previously showed that the chemokine receptor CXCR3 plays a critical role in mediating resistance to cutaneous leishmaniasis caused by *Leishmania major*. Furthermore, T cells from *L. major*-susceptible BALB/c but not resistant C57BL/6 mice fail to efficiently up-regulate CXCR3 upon activation. We therefore examined whether transgenic expression of CXCR3 on T cells would enhance resistance to *L. major* infection in susceptible BALB/c mice. We generated BALB/c and C57BL/6 transgenic mice, which constitutively overexpressed CXCR3 under a CD2 promoter, then examined their outcome to *L. major* infection. Contrary to our hypothesis, transgenic expression of CXCR3 on T cells of BALB/c mice resulted in increased lesion sizes and parasite burdens compared to WT littermates, after *L. major* infection. Re-stimulated lymph node cells from *L. major*-infected BALB/c-CXCR3<sup>Tg</sup> mice produced more IL-4 and IL-10, and less IFN-γ. Cells in draining lymph nodes from BALB/c-CXCR3<sup>Tg</sup> mice showed enhanced Th2- and reduced Th1-cell accumulation associated with increased neutrophils and inflammatory monocytes. However, monocytes displayed an immature phenotype which correlated with increased parasite burdens. Interestingly, transgenic expression of CXCR3 on T cells did not impact the outcome of *L. major* infection in C57BL/6 mice, which mounted a predominant Th1 response and spontaneously resolved their infection similar to WT littermates. Our findings demonstrate that transgenic expression of CXCR3 on T cells increases susceptibility of BALB/c mice to *L. major*. 
Introduction:

Leishmaniasis affects over 12 million people worldwide and, according to recent estimates, almost 2 million people are affected annually (1). It is therefore a major global health problem and has been classified by the world health organization as a neglected tropical disease. The most common form of the disease is cutaneous leishmaniasis (CL), which is caused by *Leishmania major*, *L. tropica* or *L. aethiopica* in the Old World, and *L. mexicana* species complex or *L. (Viannia) braziliensis* species complex in the New World. CL is characterized by the development of large papular or nodular lesions at the infection site, which often become ulcerated and may persist for months or even years. In a proportion of patients, lesions can become chronic, leading to disfiguring mucosal leishmaniasis. There are currently no vaccines available for the disease, and resistance to first-line drugs becoming increasingly common (2, 3).

In murine models of CL, it is well established that protective host immunity depends on the generation and recruitment of appropriate Th1 immune cells to the site of infection. The hallmark of Th1 responses is the production of IFN-γ which activates mononuclear phagocytes, increases the production of reactive nitrogen species (RNS) and enhances parasite killing (4) (5). CXCR3 is a chemokine receptor which is preferentially expressed on Th1 cells and activated CD8⁺ T cells, and coordinates their recruitment to inflammatory sites where they exert their effector function. It is regulated by the transcription factor T-bet, a master regulator of Th1 responses. CXCR3 has been shown to be important in immunity to intracellular parasites which require a Th1 immune response for protection, including *L. major*. Genetic deletion of cxcr3 in C57BL/6 (BL/6) mice, which are naturally resistant to *L. major*, renders them susceptible to localized...
infection. Although these mice are able to generate a Th1 immune response in the draining lymph nodes, they are unable to control parasite growth in the lesion, due to defective CD4⁺ and CD8⁺ T cell migration to the site of infection. (6). More recent studies have shown that BALB/c (BC) mice, which are genetically susceptible to \textit{L. major}, are unable to efficiently express CXCR3 on their T cells despite their ability to produce comparable amounts of IFN-γ producing T cells as resistant BL/6 mice (7). Although numerous factors have been shown to govern genetic susceptibility of BC mice to \textit{L. major}, including their inability to up-regulate IL-12Rβ2 (8), more recent studies indicate that the intrinsic deficiency in up-regulating CXCR3 upon activation might contribute to susceptibility of these mice. However, it is not known whether compensating for this deficiency through transgenic expression of CXCR3 in BC T cells would confer resistance to these mice against \textit{L. major} infection.

Transgenic mouse models have been used in the study of gene function, and have significantly enhanced our understanding of numerous elements of the immune system (9-11). Transgenic gene expression in T cells has been successfully utilized to further characterize the function of genes associated with the immune response in various disease models (12-22). In this study, we generated and characterized BALB/c and C57BL/6 T cell-specific CXCR3 transgenic mice, and analyzed immune responses generated against \textit{L. major} infection. Our novel T cell-specific CXCR3 transgenic mouse lines provide useful tools in clarifying the role of CXCR3 in various infectious, autoimmune and neoplastic disease models.
Materials and Methods:

Generation of CXCR3 Transgenic mice

Mouse CXCR3 cDNA from a C57BL/6 background was kindly provided by Dr. Bao Lu (Harvard Medical School, Boston MA). A 1.1kb fragment was generated from the cDNA template containing the CXCR3 gene with EcoRI sites incorporated into the flanking regions of the PCR product. Using these restriction enzyme sites, the CXCR3 PCR fragment was cloned into the VA CD2 vector. The resulting 15kb plasmid was checked for correct insertion of the CXCR3 cDNA cassette by restriction digest analysis and the plasmid sequence was confirmed by DNA sequencing. Large scale preparation of the CXCR3Tg targeting vector (TV) plasmid DNA was performed using the Qiagen plasmid maxi kit (Qiagen, Valencia CA) and linearized by digestion with NotI restriction endonuclease. The targeting vector was run on a 0.8% agarose gel, excised, gel purified using the Qiagen gel extraction kit (Qiagen, Valencia CA) and eluted with 20μl of clean, sterile, DNase-free microinjection buffer (10 mM Tris-HCl; 0.25 mM EDTA, pH 8.0). The size, concentration, purity and integrity of the targeting vector DNA were verified by agarose gel analysis and spectrophotometry. CXCR3Tg TV was sent to Brigham and Women’s Hospital’s Transgenic Mouse Facility for microinjection into pronuclei of C57BL/6 embryos and reimplantation into pseudopregnant females. Resulting litters were transferred to The Ohio State University Animal Facility where they were screened for integration of the CXCR3 transgene.
Screening of CXCR3 Transgenic mice

CXCR3\textsuperscript{Tg} mice were screened using either southern blotting or polymerase chain reaction (PCR). For southern blot screening, genomic DNA from mouse tails was digested using \textit{HindIII}. A PCR generated 630bp DIG labeled probe (Roche Applied Science, Indianapolis IN) was used for hybridization of membrane-containing DNA. Detection of the hybridized probe was performed using the substrate CSPD (Roche Applied Science, Indianapolis IN). Chemiluminescence was detected after exposure to a FluorChem HD2 Chemiluminescent Imaging System (ProteinSimple, Santa Clara CA).

For PCR screening, two primer sets were used for detection of the CXCR3 transgene:

Primer set 1 – P1: CGTCATCTTCACGGAGAGAA, P2: TGTTGACCACATGGCTGAGT, P3: CAGACAGAATGTGGCAGGAA. Primer set 2 – P4: TCGTAGGAGAGGCTGCTTT, P5: GCGCTCTGGCTCTCTGTGTA, P6: GGTCACCTTCCCAGTCTGAGT. Genomic DNA from mouse tails was prepared and used as template for the PCR reaction. The PCR reaction was performed according to the following cycling conditions: 95°C for 3 minutes; 35 cycles of 94°C for 40 seconds, 48°C for 40 seconds and 68°C for 1 minute; 68°C for 10 minutes; hold at 4°C.

Mouse strains

C57BL/6 CXCR3\textsuperscript{Tg} mice were bred with C57BL/6 WT (Harlan Laboratories, Indianapolis IN) mice to generate BL/6 CXCR3\textsuperscript{Tg} and BL/6 CXCR3\textsuperscript{+/+} (wild type littermates) mice. C57BL/6 CXCR3\textsuperscript{Tg} mice were also backcrossed to the BALB/c background by breeding with WT BALB/c (Harlan Laboratories, Indianapolis IN) mice for 10 generations to generate BC CXCR3\textsuperscript{Tg} and BC CXCR3\textsuperscript{+/+} (wild type littermates) mice. All mice were
maintained in a pathogen-free animal facility at The Ohio State University in accordance with NIH and Institutional guidelines.

Parasites

*L. major* (LV39) parasites were maintained by serial passage of amastigotes into the footpads of BC mice. Amastigotes isolated from infected lesions were grown to stationary phase in M199 media supplemented with 10% FCS (Atlanta Biologicals, Flowery Branch GA), 100μg/ml streptomycin and 100 U/ml penicillin (Invitrogen, Carlsbad, CA), as described previously (23).

*Leishmania* infection protocol and Parasite enumeration

Infectious metacyclic promastigotes at doses of 2.0x10⁶ parasites were inoculated into the left hind footpad of CXCR3⁷g and CXCR3⁺/+ mice. Progression of the lesion was monitored using a dial-gauge micrometer (Mitutoyo, Tokyo, Japan) by measurement of the left footpad thickness in comparison with the uninfected contralateral hind footpad. Parasite loads in infected footpads were determined by limiting dilution assay in complete Schneider’s insect media (Invitrogen, Carlsbad, CA), as described previously (23, 24).

*Histoplasma* infection

CXCR3⁺/+ and CXCR3⁷g mice were infected intranasally with a sublethal inoculum (2 x 10⁵) yeasts of *Histoplasma* strain G217B. Mice were sacrificed at day 8 and single cell suspensions from lungs were analyzed for T cell infiltration by flow cytometry.
**DNFB contact hypersensitivity challenge**

CXCR3+/+ and CXCR3Tg mice were sensitized with 0.5% DNFB as described previously (25) then challenged 6 days later on the ear dermis with 0.2% DNFB. After 24 hours post challenge, ear thickness was measured using a dial-gauge micrometer (Mitutoyo, Tokyo, Japan) and compared to thickness of unchallenged ear. Subsequently, mice were sacrificed and mouse ear dorsal and ventral dermal sheets were separated and incubated dermal side down in culture media containing 2mg/ml of collagenase A and 0.5mg/ml DNase I for 2 hrs at 37°C, then passed through a 70 um strainer to obtain single cell suspensions. Infiltrating T cells were stained with CD4 and CD8 antibodies and analyzed by flow cytometry using a FACS Calibur flow cytometer (BD Biosciences, San Jose CA).

**Cytokine ELISA**

Cells from draining lymph nodes were isolated from infected CXCR3+/+ and CXCR3Tg mice and plated at a concentration of 0.3 x10^6 cells per well in duplicates in sterile 96-well tissue culture plates. Cells were stimulated with freeze-thawed *L. major* antigen (20μg/ml). Supernatants were harvested after 72 hours incubation and analyzed for the production of IL-4, IL-10 and IFN-γ by ELISA (Biolegend, San Diego CA).

**Flow Cytometry**

Single cell suspensions were prepared from draining lymph nodes of infected CXCR3+/+ and CXCR3Tg mice and stained with antibodies against T cell, neutrophil and macrophage markers, including CD3, CD4, CD8, CD11b, Gr1, F4/80, Tim3 and ST2 (Biolegend, San Diego CA). For intracellular staining, cells were restimulated with
Phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma Aldrich, St. Louis MO) in the presence of brefeldin A for 6 hours then stained with fluorescently labelled CD3 and CD4 antibodies. Cells were then fixed, permeabilized, and stained with anti-IL-4 and IFN-γ antibodies (BD Biosciences, San Jose CA). Cells were acquired in a FACS Calibur flow cytometer (BD Biosciences, San Jose CA) and analyzed using FlowJo software (Tree Star, Inc.).

**Statistical Analysis**

Student's unpaired t test was used to determine the significance of differences between data sets. P values of P<0.05 were considered statistically significant.
Results:

Generation of T cell-specific CXCR3 transgenic mice

We designed a CXCR3 transgenic targeting vector (CXCR3\textsuperscript{Tg} TV) by inserting the mouse CXCR3 cDNA into a human CD2 minigene-based vector for T cell-specific expression in transgenic mice (26) (Figure 1A). A southern blot strategy was designed to determine integration of the targeting vector into mouse genomic DNA after microinjection of linearized CXCR3\textsuperscript{Tg} TV into the pronuclei of C57BL/6 embryos and re-implantation into pseudo-pregnant females. As shown in Figure 1B, 2 mice (15.3%) were positive for the 1.2kb transgene fragment after southern blot analysis of genomic DNA obtained from mouse tails (Figure 1B). The mouse with a higher copy number (n = 7) of the transgene was used for subsequent breeding. The resulting T cell-specific CXCR3 transgenic mice (BL/6 CXCR3\textsuperscript{Tg}) were viable and healthy with no phenotypic defects. In order to generate a T-cell specific CXCR3-transgenic \textit{L. major}-susceptible model, BL/6 CXCR3\textsuperscript{Tg} mice were backcrossed for 10 generations by breeding with WT BALB/c mice to generate BALB/c T cell-specific CXCR3-transgenic mice (BC CXCR3\textsuperscript{Tg}). Finally, we designed a PCR strategy to genotype progeny mice that contained the CXCR3 transgene and allowed for quicker identification of CXCR3\textsuperscript{Tg} mice. PCR results showed a 295bp CXCR3\textsuperscript{Tg} band or a 558bp Wild type (CXCR3\textsuperscript{+/+}) band for primer set 1, and a 402bp CXCR3\textsuperscript{Tg} band or a 267bp CXCR3\textsuperscript{+/+} band for primer set 2 (Figure 1C).
Analysis of CXCR3 expression in T cell-specific CXCR3 transgenic mice

As demonstrated from previous work by us and others, CXCR3 is expressed in only a very small percentage of T cells in the spleen and lymph nodes of naïve mice (7, 27), and is usually restricted to memory or innate T-cell subsets (28). Our CXCR3^Tg TV construct was designed for constitutive and optimal expression of CXCR3 in all T cells (26). We therefore confirmed whether CXCR3 protein is expressed constitutively in all T cells of CXCR3^Tg mice. Single cell suspensions from spleens of BL/6 CXCR3^{+/+} and BL/6 CXCR3^Tg mice were prepared and stained with fluorescently labeled anti-CD3 and anti-CXCR3 antibodies then analyzed by flow cytometry. Analysis of T cells from spleens of BC CXCR3^Tg and BL/6 CXCR3^Tg mice showed expression of CXCR3 by virtually all T cells (Figure 1D). Our data also confirms enhanced CXCR3 expression in T cells in spleens and lymph nodes of BL/6 WT mice compared to BC WT mice (7) (Figure 1D). Further, phenotypic analysis of BC and BL/6 CXCR3^Tg mice did not reveal any defect in the functions and proportions of lymphocytes in the spleen and lymph nodes when compared to CXCR3^{+/+} mice (data not shown).

Next we determined whether T cells from CXCR3^Tg mice were able to migrate efficiently in response to CXCR3 ligands. We used 2,4-dinitrofluorobenzene (DNFB) as a model of contact hypersensitivity, which induces the production of CXCR3 ligands at the challenge site (29), thereby providing a suitable model to test T cell migration in vivo. We observed significantly increased ear thickness in DNFB challenged CXCR3^Tg mice over CXCR3^{+/+} mice (Figure 2A). This was accompanied by increased CD4^+ and CD8^+ T cell migration to DNFB challenged ears in CXCR3^Tg mice compared to CXCR3^{+/+} mice as revealed by flow cytometric analysis (Figure 2B). To further demonstrate the
functionality of CXCR3 expressing T cells in CXCR3\textsuperscript{Tg} mice, we analyzed T cell migration into the lungs of CXCR3\textsuperscript{Tg} and CXCR3\textsuperscript{+/+} mice infected with the fungus Histoplasma capsulatum. We previously showed that CXCR3-expressing T cells significantly increase lung infiltration after 8 to 10 days of \textit{H. capsulatum} infection (30). At day 8 post-infection, the percentage of CD8\textsuperscript{+} T cells recruited to the lungs of CXCR3\textsuperscript{Tg} mice increased by about 5 times compared to CXCR3\textsuperscript{+/+} mice (Figure 2C).

Finally, we characterized CXCR3 expression and migration in T cells of BC CXCR3\textsuperscript{Tg} mice. Similar to BL/6 CXCR3\textsuperscript{Tg} mice, CXCR3 was expressed in virtually all T cells of BC CXCR3\textsuperscript{Tg} mice, and showed no defects in T cell migration (Figure 2D). Taken together, our data show that CXCR3 is expressed in all T cells of BC CXCR3\textsuperscript{Tg} and BL/6 CXCR3\textsuperscript{Tg} mice, and transgenic expression of CXCR3 in T cells of BL/6 and BC mice in our model results in enhanced migration of T cells in response to inflammatory signals that induce CXCR3 ligands \textit{in vivo}.

Transgenic expression of CXCR3 in T cells of C57BL/6 mice has no effect on the outcome of \textit{L. major} infection

Using knock out mouse models, CXCR3 has been shown to be critical for resistance to \textit{L. major} in BL/6 mice (6). Interestingly, only a small percentage of CD4\textsuperscript{+} T cells express CXCR3 during \textit{L. major} infection (7). \textit{L. major} infection in the BL/6 mouse background often resolves but is characterized by the development of a transient lesion. We therefore hypothesized that transgenic expression of CXCR3 would increase resistance to \textit{L. major} in this background. To investigate this, BL/6 CXCR3\textsuperscript{Tg} mice and BL/6 CXCR3\textsuperscript{+/+} littermates were infected with \textit{L. major} and footpad measurements were taken weekly. Contrary to our hypothesis, the course of infection was not altered in BL/6
CXCR3$^{Tg}$ mice (Figure 3A), as no significant differences in lesion size developed between BL/6 CXCR3$^{Tg}$ and BL/6 CXCR3$^{+/+}$ mice. Parasite load assays conducted at the conclusion of the experiment detected no parasites in infected lesions. We further compared the immune responses generated by BL/6 CXCR3$^{Tg}$ and BL/6 CXCR3$^{+/+}$ against infection by *L. major*. Lymph node cells of infected mice re-stimulated by *L. major* antigen *ex vivo* yielded no detectable differences in the production of IFN-γ, IL-4, and IL-10 by BL/6 CXCR3$^{+/+}$ and BL/6 CXCR3$^{Tg}$ mice (Figure 4A-C). Taken together, our results show that transgenic expression of CXCR3 in T cells of C57BL/6 mice does not increase resistance against CL caused by *L. major* infection.

**Transgenic expression of CXCR3 in T cells increases susceptibility of BC mice to CL caused by *L. major***

Unlike WT BL/6 mice, BC mice are genetically susceptible to *L. major* infection. Previous work from our laboratory (7), coupled with data from our current study (Figure 2D), shows that BC mice have a deficiency in up-regulating CXCR3 during *L. major* infection. BC CXCR3$^{Tg}$ mice therefore provide a more suitable model to examine the effect of transgenic CXCR3 expression on resistance to *L. major*. Since these mice correct for the deficiency in CXCR3 up-regulation genetically associated with BC background, we hypothesized that transgenic T cell expression of CXCR3 in BC mice will increase resistance to *L. major*. We examined our hypothesis by infecting BC CXCR3$^{Tg}$ mice and BC CXCR3$^{+/+}$ littermates with *L. major* in their footpads and analyzing the course of infection over a period of 10 weeks. As expected, BC CXCR3$^{+/+}$ mice developed progressive lesions and were unable to control parasite growth (Figure 3B). However, contrary to our hypothesis, BC CXCR3$^{Tg}$ mice displayed increased lesion
sizes by week 6 post-infection compared to BC CXCR3\textsuperscript{+/+} mice, which was maintained up to week 10 (Figure 3B). Moreover, parasite dilution assays showed that lesions in BC CXCR3\textsuperscript{Tg} mice contained significantly more parasites than BC CXCR3\textsuperscript{+/+} littermates (Figure 3C). Our results clearly indicate that transgenic expression of CXCR3 in T cells of BC mice increases susceptibility to \textit{L. major} infection.

\textbf{L. major}-infected BC CXCR3\textsuperscript{Tg} mice display increased Th2 and reduced Th1 cytokines.

To determine possible mechanisms behind the increased susceptibility of BC CXCR3\textsuperscript{Tg} mice to \textit{L. major} infection, we analyzed cytokine production by draining lymph-node cells of infected BC CXCR3\textsuperscript{+/+} and BC CXCR3\textsuperscript{Tg} mice re-stimulated \textit{ex vivo} by \textit{L. major} antigen. BC CXCR3\textsuperscript{Tg} mice produced significantly reduced amounts of IFN-\textgreek{g}, a Th1 cytokine critical for protection against \textit{L. major}, compared to BC CXCR3\textsuperscript{+/+} mice (Figure 4D). Production of the Th2-associated cytokine IL-4 and the anti-inflammatory cytokine IL-10 were significantly elevated in CXCR3\textsuperscript{Tg} mice compared to CXCR3\textsuperscript{+/+} littermates (Figure 4E and F). These results suggest that transgenic expression of CXCR3 in T cells of BC mice contributes to decreased Th1 and increased Th2 cytokine production during \textit{L. major} infection which is associated with susceptibility in these mice.

Flow cytometric analysis on cells isolated from the draining lymph nodes of infected BC CXCR3\textsuperscript{Tg} and BC CXCR3\textsuperscript{+/+} mice further support a mechanism of increased Th2 polarization and reduced Th1 polarization in \textit{L. major} infected BC CXCR3\textsuperscript{Tg} mice. Intracellular staining of CD4\textsuperscript{+} T cells of infected mice revealed a trend towards lower production of IFN-\textgreek{g} in BC CXCR3\textsuperscript{Tg} mice although differences were not statistically
significant (Figure 5A and C). Further, expression of Tim3, a marker for Th1 cells, were significantly reduced in CD4+ T cells of BC CXCR3Tg mice, compared to BC CXCR3+/+ mice (Figure 5F). Conversely, the percentage of IL-4 producing cells was slightly increased in *L. major* infected BC CXCR3Tg mice compared to *L. major* infected BC CXCR3+/+ mice (Figure 5B and D). A similar trend was observed in the expression of the Th2-specific surface marker ST2 in CD4+ T cells of these mice (Figure 5G). Ratios of total Th1 to Th2 cells further support a more polarized Th2 and less Th1 immune response in BC CXCR3Tg mice (Figure 5E and H). Taken together, our data suggests that enhanced Th2 polarization and reduced Th1 cytokine production contribute to the increased susceptibility of BC CXCR3Tg mice to *L. major* infection.

**Transgenic expression of CXCR3 in T cells inhibits monocyte maturation during *L. major* infection of BALB/c mice**

Alterations in the cytokine profiles of infected CXCR3Tg mice led us to examine whether other leukocytes involved in the immune response to *L. major* were affected, which could affect susceptibility to CL. Analysis of myeloid-derived populations revealed an increased accumulation of neutrophils and inflammatory monocytes in the lymph nodes of BC CXCR3Tg mice infected with *L. major* compared to BC CXCR3+/+ mice (Figure 6A – C). These populations are hosts for *Leishmania* parasites, and neutrophil accumulation has been shown to contribute to the development of a Th2 response and subsequent susceptibility to *L. major* (31). Further analysis of infiltrating monocytes in the draining lymph nodes showed a diminished level of monocyte maturation in infected BC CXCR3Tg mice relative to BC CXCR3+/+ littermates, as shown by lower expression levels of F4/80 (Figure 6D and E). These results correlate with our observed increase in
IL-10 production, a cytokine known to suppress the maturation of inflammatory monocytes during *L. major* infection (32). As precursors to macrophages and dendritic cells, these monocytes have been shown to play a crucial role in susceptibility to *L. major* (33, 34). Our results demonstrate that transgenic expression of CXCR3 in T cells of BC mice alters myeloid infiltration and maturation which negatively affect the outcome of *L. major* infection in these mice.
Discussion:

This study clearly reveals that transgenic expression of CXCR3 in T cells exacerbates the course of *L. major* infection in BC mice. These results were unexpected, as previous studies using a CXCR3 knock out model showed that endogenous CXCR3 expression is a major contributing factor to resistance against *L. major* (6). Although studies using the CXCR3 deficient model were performed in BL/6 mice, it is noteworthy that transgenic CXCR3 expression on T cells of this mouse strain does not increase protection against *L. major* infection. Interestingly, unlike BC mice, BL/6 CXCR3\(^{Tg}\) mice are not more susceptible than BL/6 CXCR3\(^{++}\) littermates. The differences in disease outcome between BL/6 CXCR3\(^{Tg}\) and BC CXCR3\(^{Tg}\) mice relative to their WT littermates in the present study indicate that the role of CXCR3 in our transgenic model varies within mouse strains. The effects of mouse strain differences in resistance to CL caused by *L. major* are well established (8, 35). While BL/6 mice develop a protective Th1 response and are naturally resistant to CL caused by *L. major*, BC mice are genetically susceptible and mount a Th2 response to the disease (8, 36). Induction of CXCR3 expression *in vitro* and *in vivo* during *L. major* infection has also been shown to be variable between these two stains – BL/6 mice up-regulate CXCR3 much more efficiently than BC mice (7). It is evident that regulated CXCR3 expression in distinct subsets of activated T cells coupled with the predominant host immune response generated against *L. major* infection critically affects the outcome of murine CL.

BC mice are known for their inability to mount an effective Th1 immune response necessary for parasite eradication (8). Unlike BL/6 mice, their immune response is dominated by the generation of T helper cells which are deficient in IL-12R\(\beta2\)
expression and are unresponsive to IL-12-mediated IFN-γ production (36-38). Subsequent studies have also revealed a deficiency in the up-regulation of CXCR3 expression on activated T cells in these mice (7). Although this deficiency is associated with susceptibility to L. major, evidence suggests that this is not directly responsible for loss of resistance to the parasite, but is simply an effect of the genetic inability of BC mouse-T cells to respond to IL-12 stimulation after L. major infection (39). As such, contrary to our previous hypothesis, transgenic expression of CXCR3 in BC mouse-T cells did not reduce susceptibility to L. major infection. Indeed, the disease was exacerbated in BC CXCR3Tg mice. Our analysis of infiltrating immune cells in infected BC mice suggests that Th1 polarization was not increased by transgenic CXCR3 expression. However, based on the frequency of Th2 cells and the amounts of IL-4 production in draining lymph nodes compared to WT littermates, transgenic CXCR3 expression in activated Th2 cells appeared to have enhanced their recruitment to infected areas of the host.

In-depth analysis of cellular immune responses show that transgenic expression of CXCR3 in T cells of BC mice significantly affects the recruitment and maturation of myeloid subsets that are involved in immunity against Leishmania. The early stages of L. major infection are characterized by significant accumulation of neutrophils which is usually sustained in BC mice throughout the course of infection (40, 41). Leishmania parasites are able to evade the leishmanicidal activity of neutrophils, exploiting them as “Trojan horses” which mediate the silent entry of the parasite into mononuclear phagocytes (41-43). While the role of neutrophils on immunity to Leishmania remains controversial, numerous studies implicate early neutrophil recruitment as a major
contributor to parasite growth and replication in the host (44). *In vivo* depletion of neutrophils in BC mice has been shown to significantly reduce parasite loads (45). Other studies show that large neutrophil accumulation during *L. major* infection of BC mice contributes to the early development of Th2 immune responses, and transient neutrophil depletion hinders Th2 response development, resulting in a resistant phenotype (31). The results of our study showed that transgenic expression of CXCR3 in T cells of BC mice resulted in increased neutrophil accumulation in draining lymph nodes after *L. major* infection. This increased neutrophil accumulation evidently contributed to an enhanced Th2 immune response and subsequent higher parasitic burdens in BC CXCR3<sup>Tg</sup> mice compared to BC CXCR3<sup>+/+</sup> littermates.

During the early stages of *L. major* infection, monocytes are recruited to infection sites where they engulf parasitized and apoptotic neutrophils. Monocytes are crucial to immunity against *L. major* as precursors to macrophages and inflammatory dendritic cells (33). As primary target host cells, they also serve as reservoirs of *Leishmania* parasites. Their ability to successfully eradicate intracellular *L. major* parasites depends on their degree of maturation and activation state (34, 40, 46). Our study showed that transgenic expression of CXCR3 in T cells altered the recruitment of inflammatory monocytes during *L. major* infection of BC mice. Infected BC CXCR3<sup>Tg</sup> mice showed greater monocyte infiltration compared to BC CXCR3<sup>+/+</sup> littermates. The majority of infiltrating monocytes in infected CXCR3<sup>Tg</sup> mice displayed an immature phenotype, lacking expression of F4/80. Previous studies on experimental CL have shown that inflammatory monocytes lacking F4/80 expression are much less leishmanicidal than F4/80<sup>+</sup> mature macrophages (46). BC mice are characteristically known to produce less
F4/80+ mature macrophages than BL/6 mice, thereby facilitating parasite spread (46).

The comparably reduced frequency of mature macrophages in BC CXCR3Tg mice is undoubtedly a contributing factor to the increased susceptibility to *L. major*. The immune-modulatory cytokine IL-10 is known to inhibit the maturation of inflammatory monocytes (32). We observed enhanced IL-10 production by *L. major* antigen re-stimulated lymph node cells of infected BC CXCR3Tg mice, which could explain the increased frequency of immature monocytes in these mice. This unexpected effect of transgenic T cell expression of CXCR3 on monocyte maturation during *L. major* infection expands our current understanding of the role of CXCR3 in macrophage function (47-50).

In conclusion, we demonstrate the successful generation of a novel transgenic CXCR3 mouse model that will enable clarification of the role of CXCR3 in infectious, autoimmune and neoplastic disease. Our results show that transgenic expression of CXCR3 on T cells exacerbates CL caused by *L. major* in BC mice by amplifying Th2 host immune responses, increasing neutrophil and inflammatory monocyte infiltration to infected sites and inhibiting monocyte maturation.
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Conflict of Interest:

The authors do not have a commercial or other association that might pose a conflict of interest.
References:


Figure Legends:

Figure 1: Generation of CXCR3<sup>Tg</sup> mice.

(A) CXCR3<sup>Tg</sup> targeting vector and strategy for southern blot detection of transgenic clones. Targeting vector coding for the cxcr3 gene driven by the human CD2 promoter was injected into fertilized eggs of C57BL/6 mice (B) Southern blot screening of genomic DNA from mice obtained from re-implanted embryos containing the CXCR3 transgene. Mouse 833 was used for the generation of the transgenic line. (C) PCR screening of genomic DNA from CXCR3<sup>Tg</sup> and CXCR3<sup>+/+</sup> mice using 2 separate primer pairs. PCR products were electrophoresed on a 1% agarose gel. Arrows depict transgenic bands. WT - CXCR3<sup>+/+</sup> Tg - CXCR3<sup>Tg</sup>. (D) Flow cytometric analysis of cells isolated from the thymus, lymph nodes and spleens of BC CXCR3<sup>+/+</sup>, BL/6 CXCR3<sup>+/+</sup>, BC CXCR3<sup>Tg</sup> and BL/6 CXCR3<sup>Tg</sup> mice stained with CD3, CD4, CD8 and CXCR3 antibodies. Numbers represent percentage of CXCR3 expressing T cells. Cells are gated on CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Results are representative of three separate experiments with similar results.

Figure 2: Phenotypic characterization of CXCR3<sup>Tg</sup> mice.

(A) Ear thickness measurements of DNFB challenged CXCR3<sup>+/+</sup> and CXCR3<sup>Tg</sup> mice. Data shown are mean +/- SEM of 3 or 4 mice per group and are representative of 2 separate experiments with similar results **p < 0.01 using unpaired t test. (B) Flow cytometric analysis of CD4<sup>+</sup> and CD8<sup>+</sup> populations migrating to the ear pinnae of BL/6 CXCR3<sup>+/+</sup> and BL/6 CXCR3<sup>Tg</sup> mice following DNFB challenge. Numbers represent percentages of the total cells in the gated population. (C) Flow cytometric analysis of
migrating CD8⁺ T cell populations in lung infiltrates of BL/6 CXCR3⁺/⁺ and BL/6 CXCR3Tg mice challenged with a sublethal dose of *H. capsulatum*. (D) Flow cytometric evaluation of CXCR3 expression in BC CXCR3⁺/⁺ and BC CXCR3Tg mice in draining lymph nodes following challenge with *L. major* parasites. All plots shown are representative data from at least 2 separate experiments of 5 to 10 mice per group with similar results.

**Figure 3: Effect of transgenic T cell expression of CXCR3 on lesion growth of CL in BL/6 and BC mice.**

Footpad lesion measurements of CXCR3⁺/⁺ and CXCR3Tg mice infected with *L. major* in (A) C57BL/6 and (B) BALB/c backgrounds. (C) Parasite burden quantification in BC CXCR3⁺/⁺ and BC CXCR3Tg mice as determined by parasite dilution assay. Data represent mean ± SEM of 9 or 10 mice per group and are representative of 2 separate experiments with similar results. *p < 0.05, **p < 0.01, ***p < 0.001 using student’s unpaired t test.

**Figure 4: Cytokine profiles of BALB/c and C57BL/6 CXCR3Tg mice infected with *L. major*.**

(A - C) Concentration of (A) IFN-γ, (B) IL-4, and (C) IL-10 produced by cells isolated from draining lymph nodes of *L. major* infected BL/6 mice re-stimulated with *L. major* antigen as determined by cytokine ELISA. (D – F) Concentration of (D) IFN-γ, (E) IL-4, and (F) IL-10 produced by cells isolated from draining lymph nodes of *L. major* infected BALB/c mice re-stimulated with *L. major* antigen as determined by cytokine ELISA. Data represents mean ± SEM of duplicates from 5 to 10 individual mice per group and are
representative of two independent experiments with similar results. *p < 0.05 and **p < 0.01 using student’s unpaired t test.

Figure 5: Th1 and Th2 responses of *L. major* infected BALB/c CXCR3Tg mice.

(A) Intracellular flow cytometric analysis of IFN-γ producing CD4+ T cells in lymph nodes of *L. major* infected BC CXCR3+/+ and BC CXCR3Tg mice. Plots are representative data from 3 mice per group with similar results. (B) Intracellular flow cytometric analysis of IL-4 producing CD4+ T cells in lymph nodes of *L. major* infected BC CXCR3+/+ and BC CXCR3Tg mice. Plots are representative data from 3 mice per group with similar results. (C) Percentages of IFN-γ producing CD4+ T cells in lymph nodes of *L. major* infected BC CXCR3+/+ and BC CXCR3Tg mice. (D) Percentages of IL-4 producing CD4+ T cells in lymph nodes of *L. major* infected BC CXCR3+/+ and BC CXCR3Tg mice. (E) Ratio of total IFN-γ to IL-4 producing T cells in lymph nodes of *L. major* infected BC CXCR3+/+ and BC CXCR3Tg mice based on intracellular staining with IFN-γ and IL-4 antibodies. (F) Percentages of Th1 cells in lymph nodes of *L. major* infected BC CXCR3+/+ and BC CXCR3Tg mice. Cells were stained with Tim3 antibody and data are presented as percentages of CD4+ cells. (G) Percentages of Th2 cells in lymph nodes of *L. major* infected BC CXCR3+/+ and BC CXCR3Tg mice. Cells were stained with ST2 antibody and data are presented as percentages of CD4+ cells. (H) Ratio of total Th1 to Th2 cells in lymph nodes of *L. major* infected BC CXCR3+/+ and BC CXCR3Tg mice based on Tim3 and ST2 antibody staining. All graphs represent mean ± SEM of 3 individual mice per group. **p < 0.01 using student’s unpaired t test.
Figure 6: Analysis of myeloid cell populations in BALB/c CXCR3<sup>Tg</sup> mice infected with <i>L. major</i>.

(A) Flow cytometric analysis of neutrophil and monocyte populations in draining lymph nodes of <i>L. major</i> infected BL/6 CXCR3<sup>+/+</sup> and BL/6 CXCR3<sup>Tg</sup> mice. Plots are representative data from 3 mice per group with similar results. (B and C) Percentages of (B) neutrophils and (C) monocytes in draining lymph nodes of <i>L. major</i> infected BC CXCR3<sup>+/+</sup> and BC CXCR3<sup>Tg</sup> mice. Data represent mean ± SEM of 3 individual mice per group. (D) Contour plots showing percentages of F4/80<sup>+</sup> macrophages in lymph nodes of BC CXCR3<sup>+/+</sup> and BC CXCR3<sup>Tg</sup> mice infected with <i>L. major</i>. Cells are gated on monocyte populations shown in Figure 6A. Plots are representative data from 3 mice per group with similar results. (E) Percentages of total monocytes which are F4/80<sup>+</sup> in lymph nodes of BC CXCR3<sup>+/+</sup> and BC CXCR3<sup>Tg</sup> mice infected with <i>L. major</i>. Data represent mean ± SEM of 3 individual mice per group. *p < 0.05 using student's unpaired t test.