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Importance of the CCR5-CCL5 axis for mucosal *Trypanosoma cruzi* protection and B cell activation[1]

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Abstract

Trypanosoma cruzi is an intracellular parasite and the causative agent of Chagas disease. Previous work has shown that the chemokine receptor CCR5 plays a role in systemic *T. cruzi* protection. We evaluated the importance of CCR5 and CCL5 for mucosal protection against natural oral and conjunctival *T. cruzi* challenges. *T. cruzi*-immune CCR5$^{-/-}$ and wild-type C57BL/6 mice were generated by repeated infectious challenges with *T. cruzi*. CCR5$^{-/-}$ and WT mice developed equivalent levels of cellular, humoral and protective mucosal responses. However, CCR5$^{-/-}$-immune mice produced increased levels of CCL5 in protected gastric tissues, suggesting compensatory signaling through additional receptors. Neutralization of CCL5 in CCR5$^{-/-}$-immune mice resulted in decreased mucosal inflammatory responses, reduced *T. cruzi*-specific antibody secreting cells, and significantly less mucosal *T. cruzi* protection, confirming an important role for CCL5 in optimal immune control of *T. cruzi* replication at the point of initial mucosal invasion. To further investigate the mechanism responsible for mucosal protection mediated by CCL5-CCR5 signaling, we evaluated the effects of CCL5 on B cells. CCL5 enhanced proliferation and IgM secretion in highly purified B cells triggered by suboptimal doses of LPS. In addition, neutralization of endogenous CCL5 inhibited B cell proliferation and IgM secretion during stimulation of highly purified B cells, indicating that B cell production of CCL5 has important autocrine effects. These findings demonstrate direct effects of CCL5 on B cells, with significant implications for the development of mucosal adjuvants, and further suggest that CCL5 may be important as a general B cell co-activator.

Introduction

Trypanosoma cruzi is an intracellular parasite and the causative agent of Chagas disease, affecting approximately 8–11 million people in Latin America (1) of which ~10–40% develop chronic cardiac and/or gastrointestinal complications. Transmission of *T. cruzi* can occur cutaneously through exposure to parasites present in the reduviid vector excreta contaminating the reduviid bite site. Transmission also occurs through mucosal infection

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after oral and conjunctival exposure to infected reduviid excreta. In addition, *T. cruzi* can be transmitted through blood and organ donation from infected individuals. Although Chagas disease predominantly affects individuals in Latin America, an estimated 300,000 immigrants from endemic countries are chronically infected with *T. cruzi* and can transmit the parasite through blood and organ donation in the United States (2). Due to these risks, the WHO has established a new global effort to eliminate Chagas disease through prevention and control practices. As part of this new global initiative, it is important that a prophylactic and/or therapeutic vaccine against *T. cruzi* be generated in order to fully protect all those at risk of infection.

Chemokines interact with G-protein coupled receptors on leukocytes and are divided into four families (C, CC, CXC, CX3C) based on the position of the cysteine residues (3). Chemokines play important roles in both homeostatic and inflammatory conditions. CCR5 is a chemokine receptor expressed on subpopulations of lymphocytes, monocytes/macrophages, and NK cells, as well as endothelial and other nonhematopoietic cells (4–9). CCR5 is positively regulated by IL-12 (10), IFN-γ, TNF-α and IL-10 (11). $CCR5^+$ cells migrate to both mucosal and systemic sites in response to the chemokines CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES). These ligands have been shown to preferentially attract activated and memory $CD4^+$ and $CD8^+$ T cells (12–14) due to the increased level of CCR5 expressed on these cells. CCL5 (RANTES) is a chemokine produced mainly by T cells, platelets, macrophages, endothelial and epithelial cells (15). CCL5 recruits T cells, dendritic cells, monocytes, NK cells and other cell types (16) to sites of inflammation and infection due to the cell surface expression of CCR1, CCR3 and/or CCR5. The CCR5-CCL5 ligand axis (the central signaling pair representing the predominant effects of interactions between CCL3, CCL4 and CCL5 and CCR1, CCR3 and CCR5) has been shown to play a role in lymphocyte activation (17–20), differentiation (21), polarization (22–25) and survival (26). CCL5 can induce T cell adhesion to VCAM-1, ICAM-1, laminin, collagen and fibronectin proteins in the extracellular matrix (27). CCL5 also plays a role in the initiation and enhancement of antigen-specific humoral and cellular immune responses through the activation of helper T cells which enhance B cell responses and the function of antigen presenting cells (28–30).

CCR5 and CCL5 have been studied in patients with Chagas disease and in systemic models of *T. cruzi* infection in mice. High numbers of $CCR5^+$ T cells and levels of CCL5 mRNA and protein have been identified in the hearts of *T. cruzi* infected mice (31–34). Macrophages infected with *T. cruzi* have been shown to produce CCL5 mRNA and protein (35). CCL5 has been shown to induce the uptake and destruction of *T. cruzi* in macrophages in a nitric oxide-dependent manner (36, 37). $CCR5^{-/-}$ *T. cruzi* infected mice develop increased levels of blood parasitemia and acute cardiac parasitism that appears to correlate with reduced survival (31, 33). Furthermore, polymorphisms affecting CCR5 expression in humans have been associated with Chagas disease progression (38, 39). These studies have evaluated the role of CCR5 and CCL5 during *T. cruzi* systemic challenges. In this current work, we report the first investigations of the importance of CCR5 and CCL5 for mucosal *T. cruzi* protection.

Materials and Methods

Mice, parasites and challenge protocols

C57BL/6J wild-type and $CCR5^{-/-}$ female mice were obtained from Jackson Laboratories (Bar Harbor, ME, (40)) and bred at Saint Louis University. Female BALB/c mice were obtained from NCI-Charles River. Mice were bred and housed under pathogen free conditions and all studies were conducted with the approval of the Saint Louis University Animal Care Committee in an AAALAC accredited facility. Genotyping to confirm the

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knockout of *ccr5* was done via PCR using primers specific for the *ccr5* exon region reported to be deleted. Primers directed at a portion of a *ccr5* intron were used as a control. All $CCR5^{-/-}$ mice were confirmed to have the *ccr5* deletion (data not shown). The Tulahuèn strain of *T. cruzi* was used throughout these studies. Insect-derived metacyclic trypomastigotes (IMT) were obtained from *T. cruzi*-infected *Dipetalogaster maximus* triatomme insects as described previously (41). Oral and conjunctival infections were done as described previously (41, 42). Mice were initially infected with 2,000 IMT or $1–2\times10^6$ CMT (cultured-derived metacyclic tryptomastigotes; described previously (42)) either orally or conjunctivally. Starting 2 weeks after the primary *T. cruzi* infection, blood was removed from all infected mice and evaluated microscopically for parasitemia until positive. *T. cruzi*-immune $CCR5^{-/-}$ and wild-type mice were generated by repeated oral or conjunctival *T. cruzi* challenges. At least 1 month after the last challenge, *T. cruzi*-immune $CCR5^{-/-}$ and wild-type mice were orally or conjunctivally challenged with 2,000–12,500 IMT. Naïve $CCR5^{-/-}$ and wild-type mice were infected in parallel as primary controls. Mice were sacrificed 3–13 days post-challenge to evaluate immune and protection responses.

Antigen-specific IgA and IgG ELISA

Serum and fecal pellets were collected from individual mice to assess *T. cruzi*-specific circulating IgG and secretory IgA (sIgA), respectively, via enzyme-linked immunosorbent assay (ELISA). Fecal pellets were collected and added to PBS + 10% FCS (Sigma, St. Louis, MO). Samples were vortexed for 15 seconds and centrifuged at 20,000 x g for 10 minutes. Nunc PolySorp Immuno plates (Rochester, NY) were coated with 10 μg/mL of T. *cruzi* lysate overnight at 4°C. Plates were then washed four times with PBS-Tween (PBS-T) and blocked with PBS + 10% FCS for 2–4 hrs at room temperature. Serum and fecal extracts were serially diluted in PBS and incubated overnight at 4°C. Plates were then washed with PBS-T and incubated with either goat anti-mouse IgG-HRP or goat anti-mouse IgA-Biotin (SouthernBiotech, Birmingham, AL) diluted in PBS and incubated overnight at 4°C. Plates were washed and streptavidin-HRP (Jackson ImmunoResearch, West Grove, PA) was added in PBS and incubated for 90 min at room temperature. Plates were washed and developed by the addition of 3,3′,5,5′-tetramethylbenzidine substrate (Sigma, St. Louis, MO). ELISA plates were analyzed at 450 nm with a reference of 540 nm.

ELISPOT assay to assess IFN-γ, IgG and IgA producing cells

Millititer HA 96-well microtiter plates with nitrocellulose bases (Millipore, Bedford, MA) were coated with 10μg/mL of either a monoclonal antibody specific for murine IFN-γ (clone R46A2; Pharmingen, San Diego, CA) or recombinant *trans*-sialidase (rTS) (43) for up to 72 hrs at 4°C. Plates were washed with PBS four times and blocked with RPMI + 10% FCS at room temperature for at least 2 hrs. For IFN-γ ELISPOT analyses, spleen cells (5×10^5 cells/well) harvested on days 11, 12 and 13 post-challenge were added. Wells were stimulated with media alone, 10 μg/mL *T. cruzi* lysate, 10 μg/mL rTS, or 2.5 μg/mL of $H\text{-}2K^b$-restricted CD8 epitopes TSSA (ANYNFTLV (44, 45)), 77.2 (VDYNFTIV (46)) or ASP2 (VNHRFTLV (47)) (Sigma, St. Louis, MO) at 37°C in 5% CO_2 overnight. For IgG and IgA ELISPOT analyses, 1×10^6 spleen cells were added to rTS coated plates and incubated overnight at 37°C, 5% CO_2. Plates were washed with water, followed by PBS and incubated with either rat anti-mouse IFN-γ biotin (clone XMG1.2; BD Biosciences, San Diego, CA); goat anti-mouse IgA-biotin or goat anti-mouse IgG-biotin (SouthernBiotech, Birmingham, AL) for 2 hrs at room temperature in PBS or PBS+10% FCS, respectively. Plates were washed four times with PBS-T and streptavidin-HRP (Jackson ImmunoResearch, West Grove, PA) added for 2 hrs at room temperature in PBS. Plates were then washed three times with PBS-T and PBS and spots developed via 3-amino-9-ethylcarbazole substrate (AEC) precipitation. Spots were counted using a Cellular Technology Immunospot plate

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reader and scanning software (CTL, Cleveland, OH), and the results are reported as the number of spot-forming cells or antibody-secreting cells per million spleen cells.

Quantification of mucosal *T. cruzi* replication

Quantitative PCR (qPCR) and limiting dilution assays (LDA) were used to quantitate the levels of mucosal *T. cruzi* replication. *T. cruzi* challenged mice were individually assayed for both qPCR and LDA 11 to 13 days after the final IMT challenge. *T. cruzi*-specific qPCR assays were completed as described previously for both orally challenged (41) and conjunctivally challenged mice (42). In some instances, gastric tissue was placed in either RNAlater or Allprotect tissue reagent (Qiagen, Valencia, CA) in order to isolate both DNA and RNA. Tissue from the entire stomach of individual mice was homogenized and DNA/RNA isolated as per the AllPrep DNA/RNA or DNA/RNA/Protein kit manual (Qiagen, Valencia, CA). For parasite outgrowth LDA, spleen and draining lymph node cells from orally challenged mice (gastric lymph node) and conjunctivally challenged mice (submandibular/parotid lymph nodes) were serially diluted and plated in 96-well microtiter plates in parasite axenic medium as described previously (48). Plates were incubated at room temperature for 2.5 months and inspected by inverted light microscopy for parasite outgrowth of *T. cruzi* epimastigotes. Results are reported as the number of parasites per million cells.

In vivo neutralization of CCL5 in CCR5$^{-/-}$ *T. cruzi*-immune mice

A neutralizing CCL5 antibody (clone R6G9) was generated as described previously (49). We confirmed the neutralizing activity of this CCL5 antibody using an *in vitro* CCL5 transwell migration assay (data not shown). Beginning four days prior to oral *T. cruzi* rechallenge, CCR5$^{-/-}$ *T. cruzi*-immune mice were injected intraperitonally with 250 μg of either anti-CCL5 antibody or an IgG1κ isotype control antibody (Sigma, St. Louis, MO) suspended in 100 μl of sterile PBS. Mice were reinjected with the same antibodies every other day (d-4, d-2, d0, d2, d4, d6, d8, d10) and rechallenged orally with 1000–2000 IMT on d0. Twelve days after rechallenge, mice were sacrificed and assayed for immune and protective responses.

Gene expression profiling in the gastric mucosa of *T. cruzi*-immune mice

CCR5$^{-/-}$, wild-type C57BL/6, and BALB/c *T. cruzi*-immune mice were sacrificed either the day of challenge or on days 3, 7 or 14 after oral IMT challenge, gastric tissue placed in RNAlater (Qiagen, Valencia, CA) and RNA isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA). Anti-CCL5 or IgG1κ isotype control treated CCR5$^{-/-}$ *T. cruzi*-immune mice were sacrified 12 days post oral IMT challenge, gastric tissue placed in RNAlater (Qiagen, Valencia, CA) and RNA isolated using the AllPrep DNA/RNA isolation kit (Qiagen, Valencia, CA). Naïve, age-matched CCR5$^{-/-}$ or wild-type gastric tissue were used as controls. Genomic DNA was removed using the TURBO DNA-*free*™ kit (Ambion, Austin, TX) and RNA was cleaned using the RNeasy Mini Kit (Qiagen, Valencia, CA). One microgram of RNA was used to generate cDNA via the enhanced avian RT first strand synthesis kit (Sigma, St. Louis, MO). *CCL4, CCL5, CCL19, CCL22, CCL25, CXCL5, CXCL9, CXCL10, CXCL11* and *CXCL12* Taqman gene expression primer/probe sets were purchased from Applied Biosystems (Foster City, CA). *Foxp3* and *Gapdh* primers and probes were designed by the Trudeau Institute (Saranac Lake, NY) and generated by Integrated DNA Technologies (Coralville, IA). *IgA* primers and probe were designed using the RealTime PCR design tool and generated by Integrated DNA Technologies (Coralville, IA). The sequences of the *foxp3, gapdh and IgA* primers and probes are as follows: *foxp3* forward primer (5′-CCCAGGAAAGACAGCAACCTT-3′), reverse primer (5′-TTCTCACAACCAGGCCACTTG-3′), probe (5′-ATCCTACCCACTGCTGGCAAATGGAGTC-3′); *gapdh* forward primer (5′-

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CTCGTCCCGTAGACAAAATGG-3′), reverse primer (5′-AATCTCCACTTTGCCACTGCA-3′), probe (5′-CGGATTTGGCCGTATTGGGCG-3′); *IgA* forward primer (5′-GCGAGCTTTCAACCCTAA-3′), reverse primer (5′-ACACTAGGTAGCTTTCTGGG-3′), probe (5′-TGCGATGGCTGCATGGAAATGA- 3′). The Taqman gene expression master mix was used and samples were run in an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA) using the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data represent fold changes in gene expression compared to age-matched naïve wild-type or $CCR5^{-/-}$ mice calculated using the $2^{-}{}_{\Delta\Delta}{}^{Ct}$ method with *gapdh* as a housekeeping gene control. Negative controls without added reverse transcriptase were included to confirm the removal of gastric DNA.

In vitro B cell stimulation

B cells were purified from the spleens of naïve C57BL/6 mice using the EasySep negative selection mouse B cell enrichment kit (Stemcell Technologies, Vancouver, British Columbia). Typically, B cell purity was > 95% as measured by flow cytometry. In order to further purify the B cells, we performed complement-mediated T cell depletion. Purified B cells were adjusted to 1×10^7 cells/mL in RPMI 1640 + 5% FCS, 50 U/mL Penicillin, 50 μg/mL Streptomycin, 2 mM L-glutamine and 50 μM 2-mercaptoethanol, and anti-Thy1.2 antibody (5 μg/mL; BD biosciences, San Diego, CA) was added for 45 min at 4°C. Cells were washed three times and Low-Tox®-M Rabbit Complement (Cedarlane, Burlington, NC) was added at a final dilution of 1:15. Cells were incubated for 1 hr at 37°C, washed three times, counted and adjusted to 1×10^6 cells/mL in RPMI 1640 + 10% FCS, Penicillin/Streptomycin, L-glutamine and 2-mercaptoethanol. 1×10^5 highly purified B cells (> 98% $CD19^+$) were added to 96 well flat bottom plates. Lipopolysaccaride (LPS; 0111:B4; Sigma Aldrich, St. Louis, MO), recombinant mouse CCL5 (R&D Systems, Minneapolis, MN), anti-CCL5 mAb (gift from Thomas Lane, UC-Irvine) and IgG1κ isotype control mAb (Biolegend, San Diego, CA) were added as indicated in each figure legend. Cells were incubated at 37°C, 5% CO_2 for 2–7 days. In order to functionally assess the depletion of T cells in highly purified B cell cultures, total spleen cells and purified B cells (1×10^5 cells/well) were added to anti-CD3/anti-CD28-coated (1 μg/mL each; BD biosciences) 96 well U-bottom plates for 2–7 days. Supernatants were collected for CCL5 and total IgM measurement, followed by the addition of 1 μCi/well [^{3}H] thmidine (Perkin Elmer Wallac, (Waltham, MA) for 16 hrs. Samples were harvested as described previously (43).

Flow cytometry

In some instances, cells were labeled with CFDA-SE (Invitrogen, Carlsbad, CA) to measure B cell proliferation. Briefly, highly purified B cells were washed with PBS, resuspended in warm PBS at 5×10^7 cells/mL and CFDA-SE was added at a final concentration of 1 μM for 8 min at 37°C. Ice cold media was added to stop the reaction and cells were washed several times with complete medium, counted and adjusted to 1×10^6 cells/mL. Cells were stimulated as described above. On days 3, 5 and 7 after stimulation, cells were spun down and transferred to a 96 well V-bottom plate, anti-CD16/32 (Fc block) added in DPBS + 1% FCS + 0.01% sodium azide for 15 min at 4°C, and surface stained at 4°C for 30 min using CD19 PE-Cy7, CD8 APC, CD3 Alexa 700 and CD4 Pacific Blue (all antibodies purchased from BD biosciences). Cells were washed three times, fixed with 1% formaldehyde and analyzed with a LSR-II flow cytometer (BD) and FlowJo v7 software (Tree Star, Inc., Ashland, OR).

Total IgM ELISA

Culture supernatants from B cell stimulated cultures were collected at different time points and stored at −20°C until use. 96-well maxisorp plates (Nunc) were coated with 1μg/mL of

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goat anti-mouse Ig(H+L) purified antibody, incubated overnight at 4°C, washed 4 times with PBST and blocked with PBS + 10% FCS for 2 hrs at room temperature. Purified mouse IgM was used to generate a standard curve. Different dilutions of culture supernatant were added to PBS + 10% FCS and incubated overnight at 4°C. Plates were washed 4 times with PBST, goat anti-mouse IgM HRP added (1/5000 in PBS + 10% FCS) for 2 hours at room temperature before development with 3,3′,5,5′-tetramethylbenzidine substrate (Sigma). All antibodies purchased from Southern Biotech. % suppression of total IgM was calculated as follows: $((LPS_{alone} - LPS_{antibody\ treated})/LPS_{alone}) \times 100$.

CCL5 ELISA

Highly purified B cells (>98% $CD19^+$) were cultured with medium alone, LPS (10μg/mL) or anti-CD3/CD28 (1 μg/mL each). Culture supernatants were taken at days 2, 3, 5 and 7 and frozen at −20°C until use. A DuoSet mouse CCL5/Rantes ELISA (R&D systems, Minneapolis, MN) was used to quantify CCL5 levels. 2 μg/mL of rat anti-mouse CCL5 in PBS was added to Nunc maxisorp plates and incubated overnight at 4°C. Plates were blocked with PBS + 1% BSA for 1 hour at RT. Recombinant mouse CCL5 and culture supernatants diluted 1:5 in PBS + 1% BSA were added for 2 hrs at RT. Plates were developed by sequential addition of biotinylated goat anti-mouse CCL5 antibody in PBS + 1% BSA for 2 hrs at RT, streptavidin-HRP for 20 min at RT in the dark, and 3,3′,5,5′-tetramethylbenzidine substrate (Sigma).

Statistics

Statistical analyses were performed with STATISTICA version 6, 8 or 9 software (StatSoft, Inc., Tulsa, OK). Mann-Whitney U tests or student *t* tests were used to compare responses between groups.

Results

Increased gene expression levels of several inflammatory chemokines and chemokine receptors in the gastric mucosa of *T. cruzi*-immune mice after oral *T. cruzi* challenge

Previous work has shown that several inflammatory chemokines, such as CCL3, CCL4 and CCL5 are upregulated in the hearts of *T. cruzi*-infected mice. We sought to identify whether these chemokines, as well as other inflammatory chemokines and chemokine receptors, were upregulated in the gastric mucosa of *T. cruzi*-immune mice after oral *T. cruzi* rechallenge as the gastric mucosa is the primary site of infection and parasite replication after oral infection (48). *T. cruzi*-immune mice were sacrificed on days 0, 3, 7 and 14 after oral *T. cruzi* rechallenge. Quantitative PCR was used to assess the relative mRNA levels of several chemokines and chemokine receptors in the gastric mucosa (Table I). Many of the chemokines and chemokine receptors studied were upregulated prior to rechallenge (d0) compared with naïve control mice. After oral rechallenge, the relative mRNA levels of several of these genes were even further upregulated at days 7 and 14. Most notably, CCL5 (RANTES), CCL19, CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC) and CXCR3 were highly upregulated compared with naïve, age-matched controls. These results demonstrate that after oral *T. cruzi* rechallenge, several inflammatory chemokines and chemokine receptors are upregulated in the gastric mucosa, facilitating the trafficking of lymphocytes into the gastric mucosa. Furthermore, high levels of inflammation in the gastric mucosa itself may directly enhance innate control of parasite replication.

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CCR5$^{-/-}$ mice develop equivalent levels of *T. cruzi*-specific antibodies after oral and conjunctival *T. cruzi* challenges

Based on previous work demonstrating the importance of CCR5 in systemic *T. cruzi* protection (31, 33) combined with our results demonstrating an increase in the relative levels of both CCR5 and CCL5 mRNA in the gastric mucosa of *T. cruzi*-immune BALB/c mice (Table I), we decided to evaluate whether CCR5 plays an important role in mucosal *T. cruzi* protection. We first evaluated whether immune responses in CCR5$^{-/-}$versus wild-type C57BL/6 mice were different after mucosal challenge. *T. cruzi*-specific antibody responses were measured four weeks after *T. cruzi* challenge (Fig. 1). We have previously shown that after both oral and conjunctival *T. cruzi* challenges, *T. cruzi*-specific IgG and IgA can be detected in serum and fecal extracts, respectively (42, 50). There were similar levels of *T. cruzi*-specific serum IgG after secondary oral (Fig. 1A) and conjunctival (Fig. 1B) challenge in the CCR5$^{-/-}$ and wild-type mice. We also detected similar levels of *T. cruzi*-specific fecal IgA in the CCR5$^{-/-}$ and wild-type mice after secondary oral (Fig. 1C) and conjunctival (Fig. 1D) challenge. Thus, CCR5 expression is not required for B cell activation and differentiation into *T. cruzi*-specific plasma cells.

Next, the numbers of *T. cruzi*-specific IgG and IgA antibody secreting cells (ASC) were measured in the spleens of *T. cruzi*-immune CCR5$^{-/-}$ and wild-type mice 11–13 days after mucosal *T. cruzi* rechallenge via ELISPOT. There were no significant differences in the levels of *T. cruzi*-specific IgG ASC comparing the CCR5$^{-/-}$ and wild-type *T. cruzi*-immune mice after oral (Fig. 2A) or conjunctival (Fig. 2B) rechallenge. There also were no significant differences in the levels of *T. cruzi*-specific IgA ASC (data not shown). These results confirm the ELISA data showing no significant defect in the generation of *T. cruzi*-specific IgG or IgA responses in CCR5$^{-/-}$ compared with wild-type *T. cruzi*-immune mice.

CCR5$^{-/-}$ mice generate equivalent levels of *T. cruzi*-specific IFN-γ-producing T cells

Next, we evaluated antigen-specific IFN-γ responses by T cells in CCR5$^{-/-}$ mice compared with wild-type mice in response to mucosal *T. cruzi* rechallenge. Previous work has shown that CCL5 can act through both G-protein coupled receptor (chemokine receptor) dependent and independent mechanisms to activate T cell (17). As CCL5 is a major ligand for CCR5, it was unclear whether there would be a difference in IFN-γ responses in CCR5$^{-/-}$ mice. Spleen cells (SC) harvested from *T. cruzi*-immune mice 11– 13 days after *T. cruzi* rechallenge were incubated with various peptides or proteins overnight to stimulate antigen-specific production of IFN-γ as measured by ELISPOT (Fig. 2C, D). IFN-γ spot-forming cells (SFC) were measured in response to three *T. cruzi* H-2K^b restricted CD8 T cell epitopes (ASP2, VNHRFTLV (47); TSSA, ANYNFTLV (44, 45); 77.2, VDYNFTIV (46)). All three peptides have been shown previously to be potent, if not immunodominant, epitopes in H-2^b expressing mice infected with *T. cruzi*. Purified *trans*-sialidase (TS) protein and *T. cruzi* lysate were also used to stimulate antigen-specific IFN-γ responses. 11–13 days after mucosal *T. cruzi* rechallenge, there were no significant differences comparing the frequencies of antigen-specific IFN-γ responses in CCR5$^{-/-}$ and wild-type *T. cruzi*-immune mice that had been orally (Fig. 2C) or conjunctivally (Fig. 2D) rechallenged. In the conjunctivally challenged mice (Fig. 2D), there appeared to be a slight increase in the CD8$^+$ T cell response against peptide TSSA in the CCR5$^{-/-}$ immune spleen cells. However, this difference was not statistically significant ($p = 0.127$; Mann-Whitney U Test). The most prominent antigen-specific IFN-γ responses were detected after stimulating spleen cells with *T. cruzi* lysate. In orally challenged mice (Fig. 2C), a slight decrease in the amount of *T. cruzi*-specific IFN-γ production in the CCR5$^{-/-}$-immune mice was seen, but this was not statistically significant ($p = 0.51$; Mann-Whitney U Test). We also evaluated *T. cruzi*-specific IFN-γ responses during primary oral and conjunctival *T. cruzi* infection. There was no significant difference in the number of *T. cruzi*-specific IFN-γ spot-forming cells between

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the CCR5$^{-/-}$ and wild-type primary infected mice (p = 0.275 and 0.827 evaluating *T. cruzi*-lysate specific responses after oral and conjunctival infection respectively; Mann-Whitney U test; data not shown). Overall, these results demonstrate that CCR5$^{-/-}$ mice were able to generate similar levels of *T. cruzi*-specific IFN-γ responses as compared with wild-type mice.

CCR5$^{-/-}$ mice develop equivalent levels of mucosal *T. cruzi* protection

To evaluate mucosal protection at the initial point of invasion and within the draining lymph nodes, parasite replication was assessed after primary mucosal infection and rechallenge of *T. cruzi*-immune mice in both CCR5$^{-/-}$ and wild-type mice. One of the most sensitive methods to study parasite replication is through quantitative PCR (qPCR) analyses. The gastric mucosae from orally challenged mice and the nasal cavities from conjunctivally challenged mice were removed from primary infected and *T. cruzi*-immune CCR5$^{-/-}$ and wild-type mice 11–13 days after mucosal *T. cruzi* rechallenge. In both oral (Fig. 3A) and conjunctival (Fig. 3B) *T. cruzi* challenge models, there were no significant differences in parasite replication in the CCR5$^{-/-}$ mice compared with wild-type mice as detected by qPCR in either primary control or *T. cruzi*-immune mice (Fig. 3A, B). Both CCR5$^{-/-}$ and wild-type-immune mice were significantly more protected from mucosal *T. cruzi* rechallenge compared with matched primary control challenged mice (*$p < 0.005$; Mann-Whitney U Test). Next, we evaluated parasite outgrowth through the limiting dilution of cells from the spleens and draining lymph nodes (Fig. 3C–F). In the spleens of oral (Fig. 3C) or conjunctival (Fig. 3D) primary control infected mice, there were no significant differences in the levels of detectable parasites present in the CCR5$^{-/-}$ versus wild-type mice. We could not detect any parasites in 1×10^6 spleen cells from CCR5$^{-/-}$ and wild-type *T. cruzi*-immune mice (Fig. 3C, D). In the gastric lymph nodes of orally challenged mice (Fig. 3E) or the submandibular/parotid lymph nodes in conjunctivally challenged mice (Fig. 3F), there were no significant differences in the levels of detectable parasites in the primary control infected CCR5$^{-/-}$ versus wild-type control mice. In some of the *T. cruzi*-immune mice, we could detect parasites in the draining lymph nodes of both the orally and conjunctivally challenged mice, but the results were not significantly different between the CCR5$^{-/-}$ and wild-type mice (Fig. 3E, F). Overall, these results demonstrate that CCR5 is not required for the efficient development and recall of protective *T. cruzi* immunity at sites of mucosal *T. cruzi* challenge.

CCL5 neutralization in CCR5$^{-/-}$ *T. cruzi*-immune mice results in decreased gastric inflammation and mucosal protection

We next evaluated the possibility that compensatory CCL5 signaling through other receptors in CCR5$^{-/-}$ mice could allow for the development of protective *T. cruzi* immunity. We examined chemokine gene expression levels in the gastric mucosa of *T. cruzi*-immune CCR5$^{-/-}$ and wild-type mice three days after oral *T. cruzi* rechallenge. There was a significant increase in the levels of CCL5 mRNA in the gastric mucosa of the CCR5$^{-/-}$ *T. cruzi*-immune mice compared with wild-type mice (Fig. 4A; *p <0.05; t test). These results implicated a possible CCL5-mediated compensatory mechanism that could allow for the development of equivalent levels of mucosal *T. cruzi* protection. Consistent with these results, CCL5 has been shown to play a role in the enhancement of both humoral and cellular mucosal immunity (28), and has also been shown to activate human macrophages to produce nitric oxide resulting in trypanocidal activity (36). To directly address the importance of CCL5 signaling in mucosal *T. cruzi* immunity, *T. cruzi*-immune CCR5$^{-/-}$ mice were treated with either an anti-CCL5 neutralizing monoclonal antibody (49) or IgG1κ isotype control antibody during oral *T. cruzi* rechallenge. Figure 4B demonstrates that there were significantly increased levels of *T. cruzi* DNA detectable in the gastric mucosa of CCR5$^{-/-}$ *T. cruzi*-immune mice treated with the neutralizing anti-CCL5 antibody 12 days

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after oral *T. cruzi* rechallenge (*$p < 0.05$; [Mann Whitney U test]). These results confirm that CCL5 signaling plays an important role in mucosal *T. cruzi* protection. Possible mechanisms for decreased mucosal protection include the lack of antigen-specific T cells and B cells migrating into the gastric mucosa and/or effects on innate immune cells, as CCL5 has been shown to induce trypanosomal activity in macrophages.

Next, we wanted to determine if differences in *T. cruzi*-specific T or B cell responses could explain the reduced protection seen in CCR5$^{-/-}$-immune mice treated with a neutralizing anti-CCL5 antibody. We did not detect any significant differences in *T.* cruzi-specific T cell responses in CCR5$^{-/-}$ immune mice treated with the anti-CCL5 antibody compared with mice treated with the IgG1κ isotype control antibody (data not shown). However, we did detect significant decreases in the levels of *T. cruzi*-specific IgG (Fig. 5A; *$p < 0.05$; Mann-Whitney U Test) and IgA (Fig. 5B; **$p < 0.01$; Mann-Whitney U Test) antibody secreting cells (ASC) detectable in the spleens from CCR5$^{-/-}$-immune mice treated with the neutralizing anti-CCL5 antibody. We also saw a significant decrease in the levels of *T. cruzi*-specific IgA ASC (*$p < 0.05$; Mann-Whitney U Test) and a trend for a decrease in *T. cruzi*-specific IgG ($p = 0.076$; Mann-Whitney U test) ASC in wild-type immune mice treated with anti-CCL5 (data not shown). Despite these results, we did not detect significant differences in end-point titers of *T. cruzi*-specific IgG in the serum of the CCR5$^{-/-}$ mice treated with either the anti-CCL5 or isotype control (1:2.5×10^6 for each group). These apparently discrepant results are likely due to the fact that these mice had been challenged several times with *T. cruzi* prior to CCL5 neutralization and thus had already developed parasite-specific long-term plasma cells. The half-life of serum IgG has been reported to be between 1 to 4 weeks and thus would not be affected by a short-term treatment with this neutralizing antibody. However, we identified a 4.45 fold decrease in the level of *T. cruzi*-specific IgA in the fecal extracts of anti-CCL5 treated CCR5$^{-/-}$-immune mice (1:8 dilution; * $p < 0.05$ Mann-Whitney U test; Data not shown). The half-life of serum IgA has been reported to be markedly shorter (IgA $t_{1/2}$ ~17–22 hours) than serum IgG (51) and is shed as well as proteolytically degraded over time in stool. These facts may explain why neutralization of CCL5 resulted in decreased levels of antigen-specific secretory IgA. Other than producing antibodies, B cells have been shown to play important roles as professional antigen presenting cells and cytokine producing cells able to skew immune responses (52, 53). Thus, a decrease in *T. cruzi*-specific antibody-secreting cells after CCL5 neutralization might specifically affect the ability to mount an effective immune response against *T. cruzi* through the inability of B cells to efficiently present antigen or produce cytokines. Thus, neutralization of CCL5 in CCR5$^{-/-}$ *T. cruzi*-immune mice results in decreased levels of *T. cruzi*-specific B cell responses, and decreased mucosal protection in these mice.

We further evaluated the gene expression of several chemokines and other various genes in the gastric mucosa of these CCR5$^{-/-}$-immune mice 12 days after oral *T. cruzi* rechallenge. We detected marked decreases in the levels of several inflammatory chemokines (CCL4, CCL5, CXCL9, CXCL10, CXCL11; Figure 6) in the gastric mucosa of CCR5$^{-/-}$-immune mice treated with neutralizing anti-CCL5 antibody. There was also a decrease in the level of IgA mRNA in the gastric mucosa, consistent with the ELISPOT (Fig. 5B) and ELISA data showing decreased antigen-specific IgA. Overall, these results demonstrate that treatment of CCR5$^{-/-}$ *T. cruzi*-immune mice with a neutralizing anti-CCL5 antibody resulted in decreased *T. cruzi*-specific IgG and IgA ASC, decreased gastric inflammatory chemokine responses and decreased mucosal *T. cruzi* protection. These combined results indicate that CCL5 is likely to be important for both innate and adaptive immune responses required for control of *T. cruzi* mucosal infection.

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The direct effect of CCL5 on B cells

Neutralization of CCL5 in CCR5$^{-/-}$ and wild-type *T. cruzi*-immune mice resulted in decreased levels of *T. cruzi*-specific IgA and IgG antibody secreting cells (Figure 5 and data not shown). These results are consistent with previous results (28), indicating a role of CCL5 as a possible B cell adjuvant. To date, this adjuvant activity has been attributed to the effects of CCL5 on T cells as CCL5 has been shown to activate T cells (17). CCL5 upregulates CD28, CD30 and CD40L on DO11.10 CD4^{+} T cells after ovalbumin peptide stimulation (28), demonstrating that CCL5 can directly affect T cells. However, very little has been done to evaluate a potential direct effect of CCL5 on B cells. In our studies evaluating the effect of CCL5 neutralization in CCR5$^{-/-}$ mice, we did not observe any significant difference in effector T cells (data not shown). Thus, we sought to evaluate whether CCL5 could directly activate B cells. Addition of CCL5 to suboptimal concentrations of LPS increased B cell proliferation (Fig. 7A, 7B) and IgM secretion (Fig. 7C) in highly purified B cells. B cells themselves have been shown to produce CCL5 (54) and in Figure 7D, we show that 10 μg/mL of LPS can induce detectable levels of CCL5 in culture supernatants. Neutralization of CCL5 in B cells stimulated with a high concentration of LPS inhibited B cell proliferation (Fig. 7E) and total IgM secretion (Fig. 7F), suggesting that B cell production of CCL5 acts in an autocrine manner to increase B cell proliferation and total IgM secretion. CCL5 stimulation alone did not result in any detectable changes in B cell activation (Fig. 7) suggesting that CCL5 may be important as a general B cell co-activator. We have also seen similar results using purified B cells from CCR5$^{-/-}$ mice (data not shown). These data suggest that CCL5-mediated enhancement of B cell activation does not depend on direct CCL5-CCR5 signaling. CCL5 could signal through CCR1 or CCR3 in the absence of CCR5. In any case, these findings demonstrate a direct effect of CCL5 on B cells and have implications for the use of CCL5 as a mucosal adjuvant to directly enhance B cell responses.

Discussion

The role of CCR5 during *T. cruzi* infection has been studied in both humans and mice. Initially it was identified that *T. cruzi* sero-positive patients with a CCR5 59029 A→G mutation, resulting in decreased cell surface expression of CCR5, had less cardiac symptoms compared with cardiomyopathic patients (38, 39). Studies in mice have revealed increased levels of the CCR5 ligands, CCL3, CCL4 and CCL5, in the hearts of *T. cruzi* infected mice (31–34). Treatment of mice during the acute phase of *T. cruzi* infection with *N*-terminal-methionylated RANTES (Met-RANTES), a selective CCR1 and CCR5 antagonist, resulted in decreased levels of CD4^{+} and CD8^{+} T cells migrating into the heart, decreased fibronectin deposition and increased survival (32). Furthermore, CCR5$^{-/-}$ mice have been shown to be more susceptible to *T. cruzi* infection compared with wild-type mice (31, 33). Taken together, these studies have demonstrated that CCR5 plays a role in systemic protection and cardiac inflammation during *T. cruzi* infection.

In the present report, we evaluated whether CCR5 and CCL5 played a role in mucosal protection. CCR5 and CCL5 mRNA levels were upregulated at the initial site of *T. cruzi* infection and replication in *T. cruzi* immune mice several days after oral *T. cruzi* rechallenge. Our initial studies indicated that CCR5$^{-/-}$ mice had similar levels of *T. cruzi* replication in the nasal cavities after primary conjunctival infection as compared with wild-type mice (data not shown). We then wanted to evaluate whether CCR5$^{-/-}$ mice were able to develop protective memory immunity against *T. cruzi*. CCR5$^{-/-}$ and wild- type *T. cruzi*-immune mice had similar *T. cruzi*-specific IgG, IgA and IFN-γ responses and were similarly protected against *T. cruzi* mucosal infection. These results suggested that CCR5 does not play a nonredundant critical role in mucosal *T. cruzi* protection. We then wanted to evaluate possible compensatory mechanisms that allowed the CCR5$^{-/-}$mice to develop equivalent

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levels of protective mucosal immunity against *T. cruzi*. There was an increase in CCL5 mRNA in the gastric mucosa of CCR5$^{-/-}$ *T. cruzi*-immune mice three days after oral *T. cruzi* rechallenge as compared with wild-type controls. We then hypothesized that increased levels of CCL5 may 1) increase the recruitment and/or activation of memory T cells and B cells into the gastric mucosa, or 2) increase killing of *T. cruzi* through the direct activation of macrophages. Neutralization of CCL5 in these CCR5$^{-/-}$ *T. cruzi*-immune mice did not affect antigen-specific IFN-γ production by T cells, but resulted in decreased mucosal inflammatory responses, *T. cruzi*-specific IgG and IgA secreting cells and mucosal *T. cruzi* protection. To further evaluate possible mechanisms for this decreased mucosal protection, we studied the direct effects of CCL5 on B cells. Proliferative and IgM responses triggered by suboptimal doses of LPS in highly purified B cells were enhanced by CCL5. In addition, neutralization of CCL5 in purified B cell cultures stimulated with LPS inhibited their proliferative responses. Thus, neutralization of CCL5 in CCR5$^{-/-}$ *T. cruzi*-immune mice could inhibit B cell activation (both naïve and memory B cells), decreased induction of antigen-specific plasma cells, leading to decreased mucosal *T. cruzi* protection.

Our data indicate that CCR5 does not play a nonredundant critical role in mucosal *T. cruzi* protection. One possible explanation for this finding is that CCR5 may not be involved in trafficking of lymphocytes to mucosal sites and thus CCR5 would not be important for mucosal immunity. However, many investigators have reported the presence of CCR5^{+} T cells and CCR5 ligands in mucosal tissues (55–59). In one study evaluating long-term non-progression of SIV infection in Chinese rhesus macaques, early restoration of CD4^{+}CCR5^{+} T cells into the gut correlated with decreased viral load and a positive clinical outcome (60). In children infected with *H. pylori*, there were high levels of CCR5^{+} cells detected in the gastric lamina propria, suggesting that CCR5 played a role in gastric immune responses (56). Increased levels of CCR5 ligands have been reported in the gastric mucosa during *H. pylori* infection (57–59). Overall, these studies have demonstrated that both CCR5 and CCL5 play important roles during inflammatory reactions in mucosal tissues during infection.

There is a large amount of redundancy in the chemokine and chemokine receptor system. It has been demonstrated that multiple chemokines can bind to and signal through multiple chemokine receptors. This redundancy may allow for fine control over immune responses due not only to interactions between chemokines and chemokine receptors on leukocytes, but also the effects on adhesion molecule interactions, glycosaminoglycans and different oligomerization states of ligands and their receptors (3). CCL3, CCL4 and CCL5 have been shown to signal through CCR5. In addition, CCL3 can signal through CCR1 and CCR3. Furthermore, CCL5 can signal through CCR1, CCR3 and CCR5 (61). Even with this large amount of redundancy, CCR5 has been shown to be important in systemic protection against *T. cruzi* (31–33). Since it did not appear that CCR5 played a nonredundant critical role in mucosal protection against *T. cruzi*, one could hypothesize that CCL5 signaling through CCR1 and/or CCR3 may compensate for the absence of CCR5. However, deletion of both components of the CCR5-CCL5 ligand axis does appear to have important effects on mucosal *T. cruzi* protection. We did not study the role of CCL3 or CCL4 in this study as CCL5 mRNA levels were much higher compared to CCL3 and CCL4 in the gastric mucosa of *T. cruzi*-immune mice (Table I). However, CCL3 and CCL4 may also play a role in mucosal protection against *T. cruzi*. In normal mice, CCR5 may play a role in mucosal protection against *T. cruzi*. However, the large amount of redundancy may compensate for the lack of CCR5 in the CCR5$^{-/-}$ mice.

IFN-γ, IL-12 and β2-microglobulin have been shown to play critical roles in protection against *T. cruzi* as mice deficient in these molecules fail to develop mucosal and systemic immunity (62). We have previously shown the importance of Th1 T cells in protection

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against *T. cruzi*. Mice immunized with recombinant IL-12 plus an anti-IL-4 antibody to bias for Th1 responses *in vivo* had decreased blood parasitemia levels and 100% survival after a normally lethal *T. cruzi* systemic challenge. Adoptive transfer studies showed that $CD4^+$ T cells are necessary, but not sufficient, for the development of immunity protective against *T. cruzi* challenge. However, these $CD4^+$ T cells were not required for memory immune effector functions protective against *T. cruzi* rechallenge (63). Others have shown that $CD8^+$ T cells from mice immunized with *T. cruzi* antigens can transfer systemic protection to naïve mice (64). Collectively, these results demonstrate that both type 1-skewed $CD4^+$ and $CD8^+$ T cells are necessary for the development of protective immunity, but that $CD8^+$ T cells alone are sufficient for protective *T. cruzi* effector functions.

B lymphocyte responses specific for *T. cruzi* have also been shown to be important for protection. *T. cruzi*-specific serum IgG antibodies have been shown to play a role in complement activation and lysis (65–68), opsonization (69), and antibody-dependent cell cytotoxicity (ADCC) (70). *T. cruzi* mucosal infection induces protection against rechallenge, associated with *T. cruzi*-specific IgA (48). Fecal extracts containing *T. cruzi*-specific secretory IgA from *T. cruzi*-immune mice provide protective opsonization activity both *in vitro* and *in vivo* against *T. cruzi* cellular invasion (42). In addition to the effects of antibodies secreted by B cells, the antigen presentation function of B cells has been shown to be important for *T. cruzi* protective immunity (43). $\mu MT^{-/-}$ mice which lack mature B cells developed lower numbers of $CD4^+$ and $CD8^+$ central and effector memory T cells, as well as increased susceptibility after systemic *T. cruzi* infection (71, 72). These studies have demonstrated that B cells play an important role in systemic protection against *T. cruzi* through participating in the generation of central and effector memory T cells and antibodies. Overall, $CD4^+$ T cells, $CD8^+$ T cells and B cells play critical roles in the establishment of *T. cruzi* protective immunity.

Previously, CCL5 has been shown to enhance humoral and cellular immune responses after protein immunization as well as mucosal bacterial infection (28–30). High concentrations of CCL5 have been shown to induce T cell activation through both G-protein coupled receptor (GPCR)-dependent and – independent mechanisms (17, 73). Intranasal OVA plus CCL5 immunization resulted in increased levels of OVA-specific antibodies, $CD4^+$ T cell proliferation and IL-2, IFN-γ, IL-5 and IL-6 cytokine production (28). The authors of this later report hypothesized that CCL5 could augment immune responses by enhancing antigen presentation or by activating host immune cells. Neutralization of CCL5 in mice mucosally challenged with *Streptococcus pneumoniae* resulted in decreased numbers of leukocytes in multiple tissues, decreased antigen- specific IgG and IgA responses, and decreased IFN-γ, TNF-α, IL-6, IL-4 production as well as $CD4^+$ T cell proliferation in spleen cells (29). Interestingly, there was an increased level of IL-10 production in $CD4^+$ T cells from the spleen, cervical lymph nodes and NALT harvested from anti-CCL5 treated mice after antigenic restimulation, suggesting that neutralization of CCL5 may result in conditions conducive for the generation of Th2 cells or $IL-10^+$ regulatory T cells (Tr1) (29). Neutralization of CCL5 during mucosal *Chlamydia muridarum* infection resulted in increased vaginal shedding of the bacterium up to 42 days after challenge, indicating that CCL5 is important in chlamydial immunity (30). Others have studied the use of chemokines as adjuvants to modulate immune responses during DNA vaccination. CCL5 administration during DNA vaccination has been shown to polarize $CD4^+$ T cells to become Th1 cells (74) and induce potent $CD8^+$ CTL responses (75). Our data reinforces much of these previous infectious studies in that neutralization of CCL5 in *T. cruzi*-immune $CCR5^{-/-}$ mice resulted in decreased parasite-specific IgA and IgG antibody-secreting cells in the spleen, decreased secretory IgA responses and decreased mucosal *T. cruzi* protection. We did not detect any significant defect in the frequency of IFN-γ producing antigen-specific T cells after CCL5 neutralization (data not shown). However, we evaluated antigen-specific IFN-γ responses 12

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days after oral *T. cruzi* rechallenge, as this is the optimal time point to evaluate *T. cruzi* mucosal replication. We cannot rule out a delayed onset of IFN-γ responses in the CCL5 neutralized mice, associated with reduced mucosal *T. cruzi* protection at earlier time points after rechallenge, and this should be investigated in future studies. Our data indicate that increased levels of CCL5 in the gastric mucosa plays a role in mucosal protection by the activation and/or recruitment of antigen-specific B cells, and perhaps also T cells and innate immune cells such as macrophages.

We have shown that CCL5 can directly affect B cells increasing B cell proliferation and total IgM secretion (Fig. 7). As discussed above, these effects could be useful for mucosal adjuvant effects. However, these results also suggest that CCL5 may be important as a general B cell co-activator with implications in several different fields of medicine. CCL5 has been shown to be upregulated in several autoimmune diseases, such as lupus nephritis and Rheumatoid Arthritis (76, 77). Increased levels of CCL5 in the serum and urine of lupus patients have been shown to correlate with disease severity (78, 79). Since B cells play an important role in these diseases and B cells have been shown to produce CCL5 (54), autocrine effects of CCL5 on B cells in these patients may result in increased B cell activation and the formation of autoimmune antibodies. Thus, CCL5 may be important as a general B cell co-activator that could be the target for novel treatment strategies for autoimmune and other inflammatory diseases.

In conclusion, we have shown that CCR5 does not play a nonredundant critical role in mucosal protection against *T. cruzi*. $CCR5^{-/-}$ *T. cruzi*-immune mice developed similar levels of *T. cruzi*-specific antibody, IFN-γ, and protection compared with wild-type mice. However, neutralization of CCL5 in $CCR5^{-/-}$ *T. cruzi*-immune mice resulted in decreased *T. cruzi*-specific antibody responses, decreased inflammatory chemokine gene expression in gastric mucosa, and decreased overall mucosal *T. cruzi* protection. We also demonstrate direct effects of CCL5 on B cells, suggesting a role for CCL5 as a B cell co-activator. Overall, these results indicate that CCL5 is necessary for optimal control of parasite replication in the gastric mucosa via either 1) the recruitment and/or activation of memory T cells and B cells likely by signaling through a redundant set of receptors (i.e. CCR1, CCR3 and/or CCR5), or 2) direct activation of innate immune cells allowing for parasite control in the initial site of infection.

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Abbreviations used in this paper

***T.* cruzi**	*Trypanosoma cruzi*
IMT	*T. cruzi* insect-derived metacyclic tryptomastigote
CMT	*T. cruzi* cultured-derived metacyclic tryptomastigote
MT	metacyclic tryptomastigote
TS	*T. cruzi* trans-sialidase protein antigen
TSSA	tryptomastigote small surface antigen
ASP2	amastigote surface protein 2
LPS	lipopolysaccaride

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WT	wild type
ASC	antibody secreting cells
SFC	spot forming cells
sIgA	secretory IgA
LDA	limiting dilution assay
qPCR	quantitative PCR
TcME	*T. cruzi* molecular equivalents
SC	spleen cells
LN	lymph node

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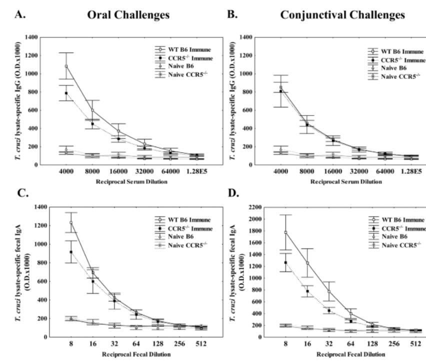

Figure 1.

CCR5$^{-/-}$ *T. cruzi*-immune mice develop similar levels of *T. cruzi*-specific serum IgG and fecal extract IgA as compared with wild-type mice. CCR5$^{-/-}$ and C57BL/6J wild-type mice were infected orally (Fig. 1A, C) or conjunctivally (Fig. 1B, D) with *T. cruzi* and challenged 8 weeks later via the same route. Four weeks later, serum (Fig. 1A, B) and fecal pellets (Fig. 1C, D) were collected from individual *T. cruzi*-immune (n=7–8/group) and naïve (n=3/group) mice, and *T. cruzi*-specific IgG (Fig. 1A, B) and IgA (Fig. 1C, D), respectively, studied via ELISA. No statistically significant differences were detected between *T. cruzi*-immune CCR5$^{-/-}$ and wild-type mice [Mann Whitney U-Test]. Immune = *T. cruzi*-immune mice; WT B6 = C57BL/6J mice

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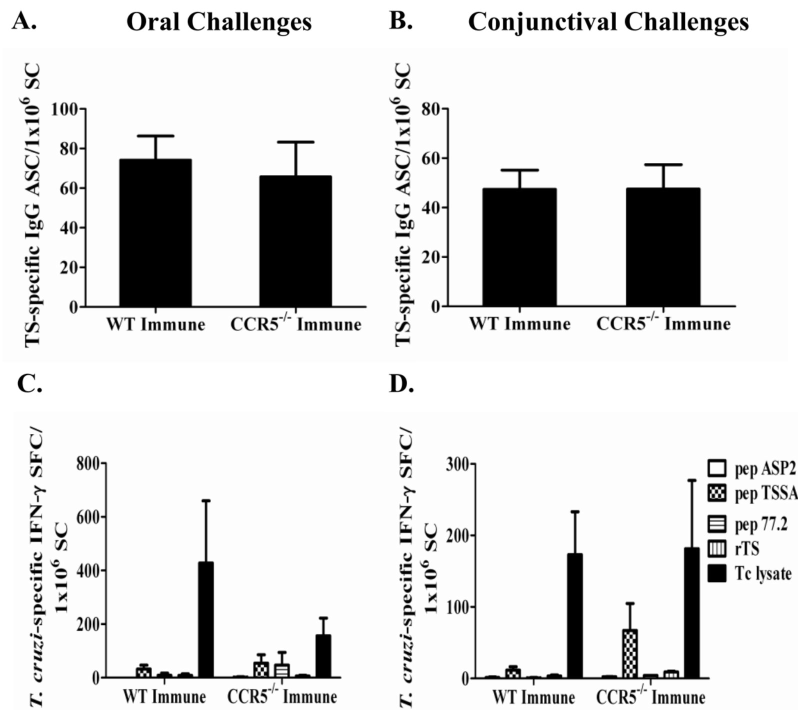

Figure 2.

CCR5$^{-/-}$ mice have similar *T. cruzi*-specific IgG and IFN-γ ELISPOT responses as compared to wild-type mice in the spleen. CCR5$^{-/-}$ and wild-type C57BL/6 mice were orally or conjunctivally challenged several times with *T. cruzi* to generate *T. cruzi*-immune mice. Eleven- to thirteen-days post *T. cruzi* rechallenge, spleen cells from orally (Fig. 2A, C) and conjunctivally (Fig, 2B, D) challenged mice were isolated and evaluated for *T. cruzi trans*-sialidase (TS) specific IgG (Fig. 2A, B) and *T. cruzi*-specific IFN-γ (Fig. 2C, D) ELISPOT responses. 1×10^6 spleen cells (SC) were added per well to assess *T. cruzi*-specific IgG ASC responses (Fig. 2A, B). In order to detect *T. cruzi*-specific IFN-γ responses, SC (5×10^5) were pulsed with *T. cruzi* (Tc) lysate (10 μg/mL), *trans*-sialidase (TS; 10 μg/mL), or three individual H-2K^b CD8 epitopes (pep ASP2, VNHRFTLV; pep TSSA, ANYNFTLV; and pep 77.2, VDYNFTIV) at 2.5 μg/mL overnight at 37°C. No significant differences were detected between *T. cruzi*-immune CCR5$^{-/-}$ and wild-type mice [Mann-Whitney U Test]. TS = trans-sialidase, ASC = antibody secreting cell, SFC = spot-forming cell, SC = spleen cells. Immune = *T. cruzi*-immune mice. WT = C57BL/6 mice. n = 7–8 mice/group.

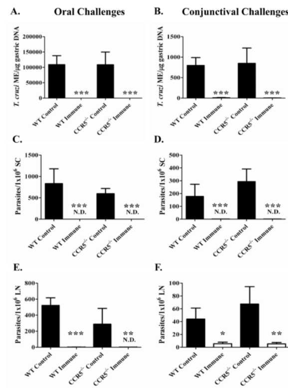

Figure 3.

CCR5 is not required for mucosal protection against *T. cruzi*. CCR5$^{-/-}$ and wild-type C57BL/6 mice were orally or conjunctivally challenged several times with *T. cruzi* to generate *T. cruzi*-immune mice. Eleven- to thirteen-days after *T. cruzi* rechallenge, mice were sacrificed, gastric DNA (Fig. 3A) and nasal cavity DNA (Fig. 3B) were isolated and the number of *T. cruzi* molecular equivalents were quantitated using a *T. cruzi*-specific qPCR assay. Spleen cells and draining lymph node cells were isolated and assessed for parasite outgrowth using a standard parasite limiting dilution assay (Fig. 3C-F). *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$; [Mann-Whitney U Test]. Control = primary infected mice, Immune= *T. cruzi*-immune mice, SC = spleen cells, ME = *T. cruzi* molecular equivalents. ND = not detectable. n = 5–8/group.

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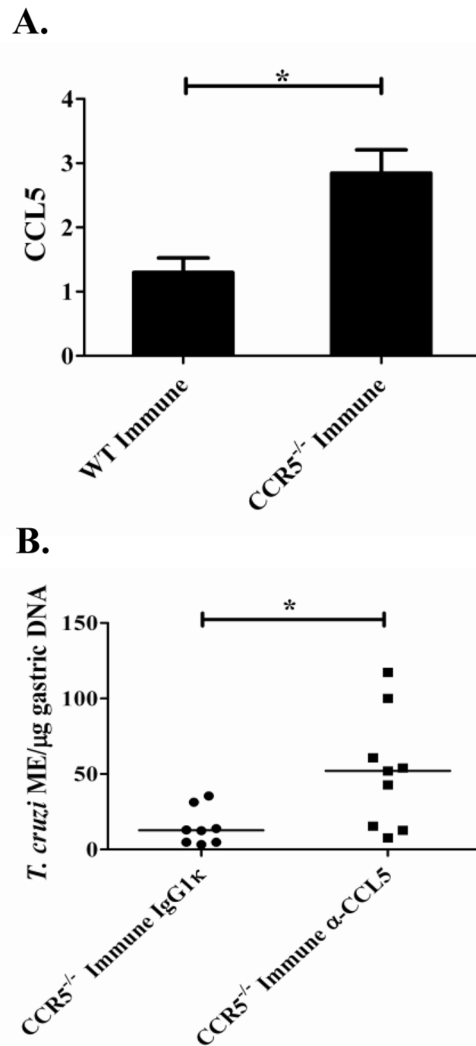

Figure 4.

CCL5 neutralization in CCR5$^{-/-}$ *T. cruzi*-immune mice leads to decreased gastric mucosal *T. cruzi* protection. CCR5$^{-/-}$ and wild-type C57BL/6 mice were orally challenged several times with *T. cruzi* to generate *T. cruzi*-immune mice. Immune and naïve mice were then orally rechallenged with *T. cruzi*. In Figure 4A, *T. cruzi*-immune mice (n=3/group), along with naïve controls, were sacrificed on day 3 and gastric RNA isolated. Data represent fold changes (± standard error) in CCL5 gene expression compared with naïve CCR5$^{-/-}$ or wild-type mice (calculated using $2^{-\Delta\Delta Ct}$ method with gapdh as a housekeeping gene). Negative controls without added reverse transcriptase were included to confirm the removal of gastric DNA (data not shown). *$p < 0.05$ [*t* test]. In Figure 4B, *T. cruzi*-immune CCR5$^{-/-}$ mice were treated with 250 μg of mouse neutralizing α-CCL5 or isotype control IgG1κ (Sigma) mAb I.P. every other day starting 4 days prior to oral *T. cruzi* challenge and continuing through day 10 after rechallenge. Total gastric DNA was isolated 12 days after rechallenge and assessed for the number of *T. cruzi* genomes (molecular equivalents; ME) using *T. cruzi*-specific real-time PCR. Lines represent median values. n=8–9/group. These results represent two independent experiments with the results pooled together. *$p < 0.05$ [Mann-Whitney U test].

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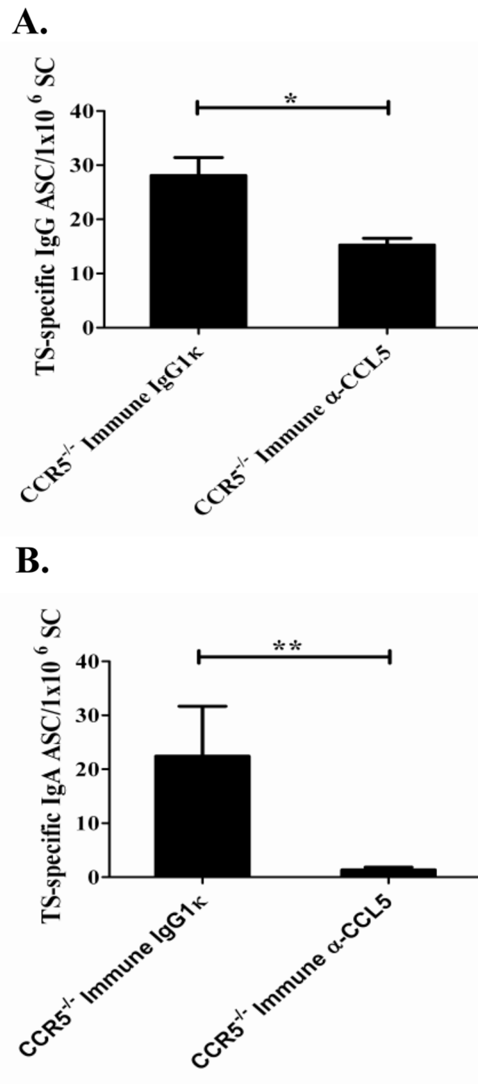

Figure 5.

CCL5 neutralization in CCR5$^{-/-}$ *T. cruzi*-immune mice leads to decreased *T. cruzi*-specific IgG and IgA antibody secreting cells in the spleen. CCR5$^{-/-}$ mice were challenged multiple times orally with *T. cruzi*. Six weeks after the last challenge, mice were treated with 250 μg of neutralizing anti-CCL5 or isotype control IgG1κ mAb I.P. and rechallenged orally with *T. cruzi* as described in Figure 4. Twelve days after rechallenge, treated CCR5$^{-/-}$ mice (n=5/ group), as well as naïve, age-matched CCR5$^{-/-}$ control mice (n=3), were sacrificed to assess *T. cruzi*-specific IgG (Fig. 6A) and IgA (Fig. 6B) antibody secreting cell (ASC) responses in the spleen. *$p < 0.05$, **$p < 0.01$ [Mann-Whitney U test]. These data represent 1 of 2 independent experiments with similar results. Immune = *T. cruzi*-immune mice; IgG1κ = IgG1κ isotype control antibody treated; α-CCL5 = anti-CCL5 neutralizing antibody treated. n=5/group.

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A.

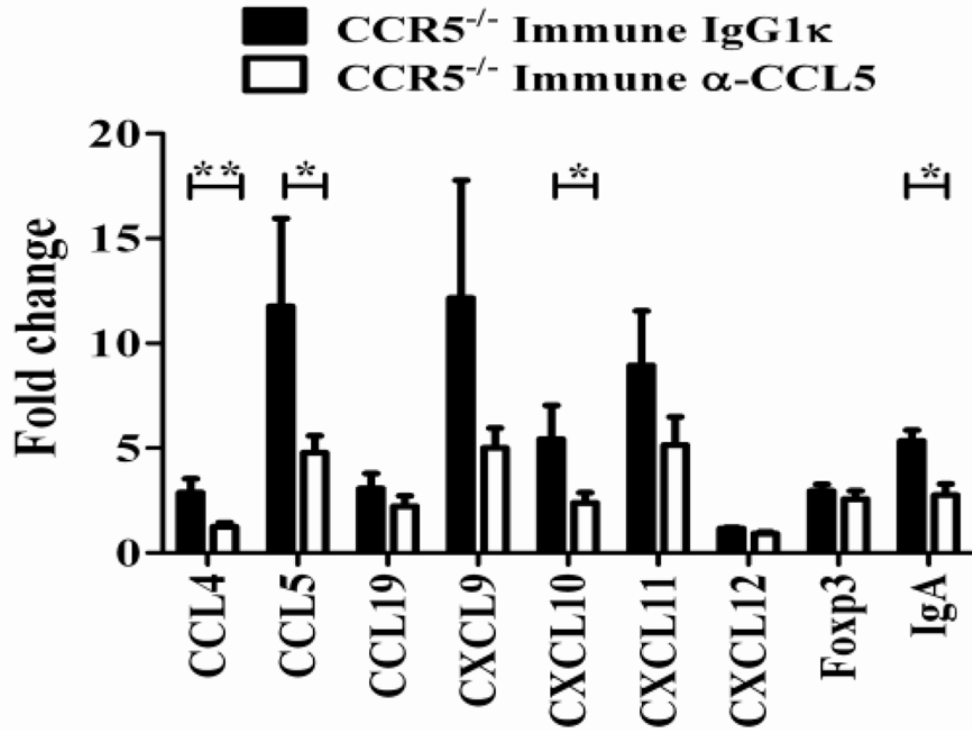

Figure 6.

CCL5 neutralization in CCR5$^{-/-}$ *T. cruzi*-immune mice leads to decreased gastric inflammatory chemokines in the gastric mucosa. CCR5$^{-/-}$ mice were orally challenged multiple times with *T. cruzi*. Six weeks after the last challenge mice were treated with 250 μg neutralizing anti-CCL5 or isotype control IgG1κ mAb I.P. and rechallenged orally with *T. cruzi* as described in figure 4. Total gastric RNA was isolated 12 days post-challenge. After genomic DNA removal and cleanup, qRT-PCR was used to measure chemokine gene expression. Data represents fold changes (± standard error) in gene expression as compared to age-matched naïve CCR5$^{-/-}$ mice (n=3) (calculated using $2^{-\Delta\Delta Ct}$ method with gapdh as a housekeeping gene). Negative controls without added reverse transcriptase were included to confirm the removal of gastric DNA. Immune = *T. cruzi*-immune, IgG1κ = IgG1κ isotype-control antibody treated. α-CCL5 = anti-CCL5 neutralizing antibody treated. *$p < 0.05$, **$p < 0.01$ [Mann-Whitney U Test]. n=5/group.

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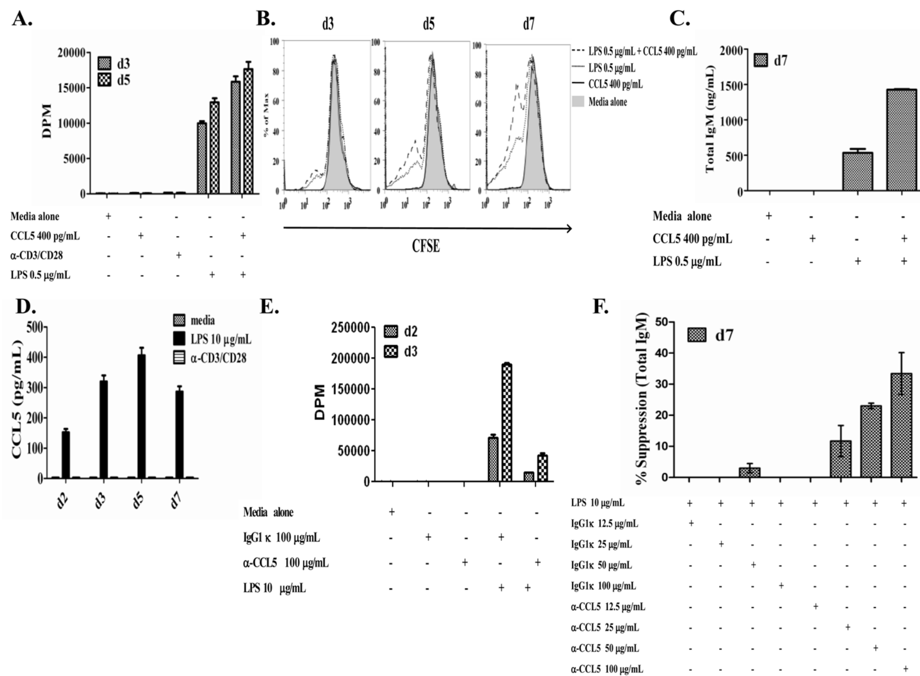

Figure 7.

The direct effects of CCL5 on B cells. Highly purified B cells (> 98% $CD19^+$) were isolated from naïve C57BL/6 mice and cultured with or without LPS (0.5 μg/mL) ± recombinant mouse CCL5 (400 pg/mL) and proliferative responses were measured via $[^3H]$-thymidine incorporation (Fig. 7A) or CFSE dilution (Fig. 7B). Culture supernatants were taken 7 days after stimulation and assessed for total IgM secretion via ELISA (Fig. 7C). Culture supernatants were taken at days 2, 3, 5 and 7 after stimulation with or without LPS (10 μg/mL) or anti-CD3/CD28, and CCL5 protein production was measured via ELISA (Fig. 7D). In a separate experiment, highly purified B cells were stimulated with or without LPS (10 μg/mL) ± anti-CCL5 mAb or IgG1κ isotype control antibody and proliferative responses measured via $[^3H]$-thymidine incorporation on days 2 and 3 post stimulation (Fig. 7E). Culture supernatants were taken 7 days after stimulation, total IgM secretion was measured via ELISA and the percent suppression was calculated $((LPS_{alone}\text{-}LPS_{antibody\ treated})/LPS_{alone}) \times 100 = \%$ suppression (Fig. 7F). Data represent two to three independent experiments with multiple triplicates of pooled samples with similar results obtained in each experiment.

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Table I

Kinetic quantification of chemokine and chemokine receptor gene expression in the gastric mucosa of *T. cruzi*-immune BALB/c mice.

	Fold change			
Chemokines	**d0 Immune**	**d3 Immune**	**d7 Immune**	**d14 Immune**
CCL3	2.55 ± 0.21*	1.18 ± 0.04	3.97 ± 0.15**	2.08 ± 0.13*
CCL4	2.31 ± 0.08**	1.45 ± 0.06	5.65 ± 0.27**	3.43 ± 0.13**
CCL5	6.63 ± 0.19***	5.12 ± 0.17***	19.62 ± 1.66**	12.00 ± 0.85**
CCL19	7.68 ± 0.84*	2.99 ± 0.23	10.57 ± 1.8*	9.36 ± 3.07*
CXCL9	2.88 ± 0.32	9.36 ± 2.79**	13.51 ± 3.11*	8.84 ± 1.17*
CXCL10	1.91 ± 0.18	4.54 ± 1.17	6.35 ± 1.27*	4.46 ± 0.38*
CXCL11	2.10 ± 0.14*	4.08 ± 0.37**	9.39 ± 2.55*	5.88 ± 0.48*
Chemokines Receptors	**d0 Immune**	**d3 Immune**	**d7 Immune**	**d14 Immune**
CCR5	1.48 ± 0.14	1.72 ± 0.05	3.30 ± 0.38*	1.76 ± 0.15
CXCR3	6.12 ± 0.59*	4.26 ± 0.25**	19.04 ± 3.88**	10.22 ± 1.52**

BALB/c mice were orally infected 3 times with *T. cruzi*. Two to four months later, mice were rechallenged (or not) with *T. cruzi* orally and gastric RNA isolated 3, 7, or 14 days later. After genomic DNA removal and cleanup, real-time PCR was used to measure chemokine and chemokine receptor expression. Data represent fold changes (± standard error) in gene expression as compared to age-matched naïve BALB/c mice (calculated using ΔΔCt method with GAPDH as housekeeping gene). Non-RT controls were added to confirm the removal of genomic DNA. N= 4–16 per group.

* $p < 0.05$,

** $p < 0.01$,

*** $p < 0.001$; [Mann-Whitney U Test] comparing ΔCt of *T. cruzi*-immune mice to the ΔCt of naïve age-matched control BALB/c mice.