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The CC Chemokine Receptor 5 Is Important in Control of Parasite Replication and Acute Cardiac Inflammation following Infection with *Trypanosoma cruzi*

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Infection of susceptible mice with the Colombiana strain of *Trypanosoma cruzi* results in an orchestrated expression of chemokines and chemokine receptors within the heart that coincides with parasite burden and cellular infiltration. CC chemokine receptor 5 (CCR5) is prominently expressed during both acute and chronic disease, suggesting a role in regulating leukocyte trafficking and accumulation within the heart following *T. cruzi* infection. To better understand the functional role of CCR5 and its ligands with regard to both host defense and/or disease, CCR5$^{-/-}$ mice were infected with *T. cruzi*, and the disease severity was evaluated. Infected CCR5$^{-/-}$ mice develop significantly higher levels of parasitemia ($P \leq 0.05$) and cardiac parasitism ($P \leq 0.01$) during acute infection that correlated with reduced survival. Further, we show that CCR5 is essential for directing the migration of macrophages and T cells to the heart early in acute infection with *T. cruzi*. In addition, data are provided demonstrating that CCR5 does not play an essential role in maintaining inflammation in the heart during chronic infection. Collectively, these studies clearly demonstrate that CCR5 contributes to the control of parasite replication and the development of a protective immune response during acute infection but does not ultimately participate in maintaining a chronic inflammatory response within the heart.

Trypanosoma cruzi is a hemoflagellate protozoan parasite that causes Chagas' disease in ca. 20 to 30% of those infected. There are approximately 18 million people in Central and South America infected with *T. cruzi*, with 100 million at risk for infection (50). The most common pathology seen in Chagas' disease is a cardiomyopathy characterized by inflammatory infiltrates, necrosis, and fibrosis (35, 45). Inflammatory infiltrates are composed primarily of CD8$^+$ T cells; however, CD4$^+$ T cells and macrophages are also commonly detected (5, 18, 34, 44, 46). Chagasic cardiomyopathy eventually results in congestive heart failure and is a major cause of death from heart failure in Latin America.

Through use of a mouse model of Chagas' disease, it has been possible to evaluate the underlying immunopathological mechanisms contributing to disease with the ultimate goal of identifying therapeutic targets for human patients. Numerous investigations have determined that various different proinflammatory cytokines including gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) are associated with inflammation and disease progression (3, 11, 33, 41, 47). More recently, there has been increased interest in characterizing the expression of proinflammatory chemokine genes within the heart following *T. cruzi* infection of susceptible mice (3, 8, 44). Chemokines and their cognate receptors direct the extravasation of leukocytes and monocytes from the bloodstream into tissues and orchestrate the positional migration of these cells within tissues. In addition, chemokines have been shown to play a significant role in the influx of cells in numerous disease models where they participate in control of pathogens and/or contribution to chronic inflammation. Chemokine genes are expressed within the hearts of *T. cruzi*-infected mice, suggesting that these molecules may have a role in defense and/or disease (3, 8, 44). In support of this idea, we and others have determined that the T-cell and macrophage chemoattractant chemokine ligands CXCL9, CXCL10, and CCL5 are detected within the heart during both acute and chronic disease (17, 44). Treatment of mice with neutralizing antibodies specific for CXCL9 and CXCL10 resulted in increased parasitemia, indicating an important role in generating a protective immune response. However, blocking these chemokines did not ultimately alter the severity of chronic disease as characterized by both parasite burden and chronic inflammation within the heart. These studies imply that the timing of chemokine gene expression is important with regards to function in this particular model. Specifically, chemokines expressed within the heart early after infection may not participate in chronic disease even though transcripts are present.

One factor controlling the response to chemokine ligand expression is the corresponding expression of the appropriate chemokine receptor(s). The goal of the present study was to characterize chemokine receptor expression within the hearts of *T. cruzi*-infected mice during acute and chronic disease. To this end, we have determined that numerous chemokine re-

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ceptors are expressed following infection with *T. cruzi*. Among the receptors detected, CC chemokine receptor 5 (CCR5) was prominently expressed during both acute and chronic disease, lending support to the possibility that signaling through this receptor may modulate cardiac inflammation and disease progression. CCR5 is expressed on a variety of cell types including lymphocytes, macrophages, granulocytes, and nonhematopoietic cells (32, 36) and is able to bind chemokines CCL3, CCL4, and CCL5 (37). After activation, both T lymphocytes and macrophages express high levels of CCR5. Inflammatory infiltrates in a number of diseases exhibit a striking increase in CCR5 expression, indicating a role for this receptor in trafficking cells to sites of inflammation (1, 4, 9, 26, 32, 40, 42). The functional role of CCR5 in the immune response during acute *T. cruzi* infection has been addressed through the use of Met-RANTES, a CCR1/CCR5 antagonist, as well as through infection of $CCR5^{-/-}$ mice (25, 27). Acute treatment with Met-RANTES resulted in decreased infiltration of T cells into the hearts of *T. cruzi*-infected mice at day 28 postinfection (p.i.) but did not alter the parasite burden (27). Cardiac inflammation was also greatly reduced in CCR5-deficient mice at day 20 p.i.; however, the parasite burden is increased during acute infection (25). Therefore, the present study was undertaken to better understand the functional role of CCR5 in both host defense and disease development following *T. cruzi* infection. In order to accomplish this, $CCR5^{-/-}$ mice were infected with *T. cruzi*, and disease progression, as well as chemokine and cytokine expression, in the heart were monitored at defined time points through acute and chronic infection. We report here that CCR5 plays a critical role during the early stages of disease by controlling parasite replication, as well as inflammation within the heart. Mechanistically, the ability of CCR5-deficient T cells to produce IFN-γ and activate macrophages, as well as the ability of CCR5-deficient splenocytes to migrate to the heart, was also examined. In addition, we characterized the role of CCR5 in maintaining inflammation in the heart during chronic infection to evaluate the potential efficacy of targeting CCR5 during chronic infection in an effort to ameliorate chronic cardiomyopathy. We provide data that support earlier studies demonstrating an important role for CCR5 in host defense after *T. cruzi* infection (25, 27). Importantly, the present study extends earlier work by demonstrating that CCR5 is not essential for T-cell-mediated cytokine secretion, nor does the absence of CCR5 attenuate the severity of cardiac inflammation during chronic disease.

MATERIALS AND METHODS

Mice. Female $CCR5^{+/+}$ mice (C57BL/6J) were obtained from The Jackson Laboratory (Bar Harbor, ME) and used at 6 to 8 weeks of age. Female $CCR5^{-/-}$ mice (eighth generation back-crossed to C57BL/6, *H-2*^{b} background) were bred and housed under specific-pathogen-free conditions in enclosed filter-top cages (39).

Parasites and infection. The Colombiana strain (10) of *T. cruzi* was maintained as previously described by serial passage in female BALB/cByJ mice (51). Mice were infected subcutaneously with 50 blood-derived trypomastigotes. Animals were euthanized on days 0, 15, 30, 60, and 120 p.i., and hearts were collected for different assays.

Parasitemia levels were determined as previously described (51) by removing a blood sample from the tail vein, diluting the sample in 0.9% ammonium chloride, and counting the trypomastigotes in a Neubauer hemacytometer (American Optical Corp., Buffalo, NY).

Cardiac tissue analysis. Hearts (three to six from each experimental group) were removed from mice at various times p.i. and fixed in a 10% formalin solution. Hearts were then paraffin embedded, and 5-μm sections were cut at 100-μm increments. Sections used for inflammation scoring and analysis of infected cardiomyocytes were hematoxylin and eosin stained. Five sections from each heart were examined. Sectional areas were determined by scanning the slides into a digital image file, followed by measurement of the tissue area with the Scion Image analysis software package. Each section was scored blindly for inflammation by using the 0 to 4 scale previously described by Sun and Tarleton (43). Briefly, the scale used was: 0, no inflammation; 1, focal inflammation; 2, multifocal inflammation; 3, diffuse inflammation with partial wall involvement; and 4, diffuse inflammation with total wall involvement. To determine the degree of tissue parasitism, infected cardiomyocytes were counted in each hematoxylin-and-eosin-stained section. Infected cardiomyocytes have been estimated to be approximately 70 μm in diameter (31); thus, we examined sections cut at 100-μm increments to reduce the probability of counting the same infected cell more than once.

RPA. Total RNA was extracted from hearts of mice at various times postinfection by using TRIzol reagent (Invitrogen, Carlsbad, CA). A minimum of three mice per time point were assayed. Chemokine receptor transcripts were analyzed by using custom multitemplate probesets (BD Pharmingen, San Diego, CA). RNase protection assay (RPA) analysis was performed with 12 μg of total RNA using a previously described protocol (22, 23). A probe for L32 was included to verify consistency in RNA loading and assay performance. For quantification of signal intensity, autoradiographs were scanned, and transcript signals were normalized as the ratio of band intensity to the L32 control (22, 23). Analysis was performed by using Scion Image analysis software package.

Macrophage activation assays. Mice were infected as described above. Fifteen days postinfection spleens were removed and single-cell suspensions were prepared in Dulbecco modified Eagle medium supplemented with 25 mM HEPES buffer (pH 7.2); 1 mM sodium pyruvate, nonessential amino acids, and L-glutamine; 5×10^{-5} M 2-mercaptoethanol; 50 U of penicillin/ml; 50 μg of streptomycin sulfate/ml; and 10% fetal bovine serum (C-DMEM). Spleen cell suspensions were enriched for T cells by passage over nylon wool columns (21). IC-21 macrophages (ATCC TIB-186) were plated in C-DMEM at 4×10^4 macrophages/well in 96-well plates. Macrophages were incubated with 4×10^5 heat-killed trypomastigotes (HKT) per well, with media (as a negative control), or 5 ng of concanavalin A (ConA)/μl (as a positive control). HKT were prepared by incubating tissue culture-derived strain Y trypomastigotes (8×10^6/ml) at 56°C for 1 h. Splenic T cells were added to the macrophage cultures at 5×10^5 T cells/well. Cells were cultured for 7 days (37°C, 8% CO_2), and supernatants were harvested at days 2, 4, and 7 and assayed for nitrite and IFN-γ production.

Nitrite assays. Nitrite levels in 2-, 4-, and 7-day culture supernatants were measured by using the Greiss reagent as previously described (28). Briefly, 50-μl culture supernatants were combined in a 96-well plate with a 1:1 mixture of 1% sulfanilamide in 2.5% H_3PO_4 and 0.1% napthylethylenediamide in 2.5% H_3PO_4. Plates were incubated for 10 min at room temperature, and the absorbance was measured at 550 nm using an automated microplate reader. Nitrite concentrations were determined in triplicate by using a standard curve of sodium nitrite from 125 to 1 μM prepared in culture media.

IFN-γ measurements. Culture supernatants were assayed for IFN-γ by capture enzyme-linked immunosorbent assay (ELISA) as previously described (29). Briefly, culture supernatants and IFN-γ standards were diluted and incubated overnight in 96-well microtiter plates coated with IFN-γ-specific capture antibody (BD Pharmingen, San Diego, CA) according to the manufacturer's recommendations. Bound cytokine was detected by using sequential incubations with biotinylated anti-IFN-γ detecting antibody, streptavidin-peroxidase, and ABTS [2,2′azinobis(3-ethylbenzthiazolinesulfonic acid); Roche Diagnostics, Indianapolis, IN]. All samples were tested in triplicate. Plates were read at 405 nm by using an automated ELISA plate reader. Concentrations were calculated from the linear regions of IFN-γ standards, and final concentrations were expressed in picograms per milliliter.

Adoptive transfer. Spleens were removed from $CCR5^{+/+}$ or $CCR5^{-/-}$ mice isolated 15 days p.i., a single-cell suspension was prepared, and red blood cells were lysed with water followed by the addition of 10× phosphate-buffered saline to restore osmotic balance. Splenocytes were adoptively transferred (2.5×10^6 cells in 100 μl of sterile Hanks balanced salt solution [HBSS]) via injection into the retro-orbital sinus of $RAG1^{-/-}$ mice 15 days after infection with *T. cruzi*. As a control, $RAG1^{-/-}$ mice, infected 15 days prior to adoptive transfer with *T. cruzi*, received 100 μl of sterile HBSS via injection into the retro-orbital sinus. Mice were euthanized at 15 days after transfer (30 days p.i.), and hearts were collected. Hearts were divided in half for histological analysis. One-half of each heart was fixed in 10% normal buffered formalin for 24 h, after which they were

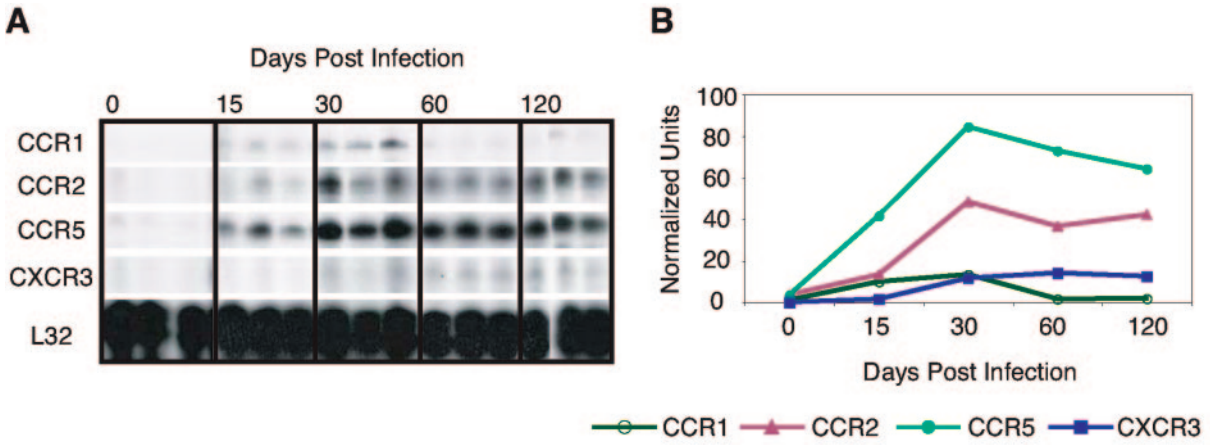

FIG. 1. C57BL/6 mice were infected with 50 trypomastigotes of the Colombiana strain of *T. cruzi*. RNA was extracted from hearts at days 0, 15, 30, 60, and 120 p.i. and used for RPA analysis of chemokine receptor expression. Shown is a representative autoradiograph (A) and average chemokine receptor expression normalized as a percentage of L32 expression (B).

paraffin embedded and stained with hematoxylin and eosin to examine inflammation and cardiac parasitism as described above. The other half of the heart was frozen in O.C.T. medium (Sakura, Torrance, CA) and stored at −80°C. Control animals received 100 μl of sterile HBSS (Mediatech, Herndon, VA) intravenously 15 days after infection with *T. cruzi* and were also euthanized at 30 days p.i.

Statistical analysis. Data are expressed as means ± the standard deviation or standard error for tissue parasitism. Statistical differences between two groups of mice were determined by the Student *t* test for RPA data, the Mann-Whitney test for histological and parasitemia data, and the Fisher exact test for survival data. *P* values of ≤0.05 were considered significant.

RESULTS

Expression of chemokines receptors in the heart of *T. cruzi*-infected mice. C57BL/6 mice were infected subcutaneously with 50 blood-derived trypomastigotes, and the expression chemokine receptors CCR1, CCR2, CCR3, CCR4, CCR5, CCR7, CCR8, and CXCR3 were measured in the heart at days 0, 15, 30, 60, and 120 p.i. by using RPA. Of the chemokine receptor transcripts measured, CXCR3, CCR1, CCR2, and CCR5 were the most highly and consistently expressed in the heart (Fig. 1A). No chemokine receptor transcripts were detected in uninfected mice (Fig. 1A). Similar expression kinetics for CCR1, CCR2, and CCR5 were revealed with transcripts detected by 15 p.i. and subsequently peaking at day 30 p.i., followed by a gradual decline out to 120 days p.i., whereas CXCR3 expression is not detected until day 30 p.i., after which its expression level remains constant through day 120 p.i. (Fig. 1B). Interestingly, the expression profile for these receptors closely paralleled the expression of chemokine ligands for these particular receptors and the timing of expression coincides with the severity of parasite burden within the heart and inflammation (17).

Increased mortality and parasitemia in CCR5$^{-/-}$ mice. Of the chemokine receptor transcripts detected within the heart, expression of CCR5 was comparatively higher and prolonged compared to others, suggesting an important role in defense and/or disease progression in *T. cruzi*-infected mice (Fig. 1A and B). To test this possibility, CCR5$^{+/+}$ and CCR5$^{-/-}$ mice were infected with 50 blood-derived trypomastigotes of the Colombiana strain of *T. cruzi*, and both survival and parasitemia were monitored out to 120 days p.i. As shown in Fig. 2A, CCR5$^{-/-}$ mice have decreased survival during acute and early chronic infection. In contrast to CCR5$^{+/+}$ mice in which 100% of animals survive to 120 days p.i., CCR5$^{-/-}$ mice began dying early (day 28 p.i.), and this gradually increased until day 59 p.i., at which point 20% of mice had succumbed to infection

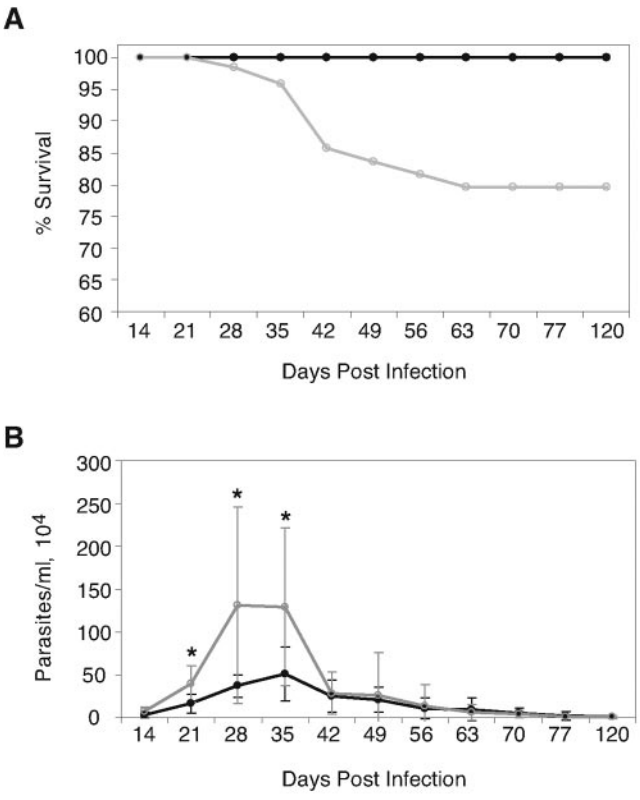

FIG. 2. Survival is decreased and parasitemia increased in CCR5-deficient mice. CCR5$^{+/+}$ (black) and CCR5$^{-/-}$ (gray) were infected with 50 trypomastigotes of the Colombiana strain of *T. cruzi*. Survival (A) and parasitemia (B) were monitored weekly from days 14 to 77 p.i. and at day 120 p.i. Survival is significantly lower in CCR5$^{-/-}$ mice, as determined by the Fisher exact test ($P \leq 0.01$). An asterisk denotes a significant difference, as determined by the Mann-Whitney test ($P \leq 0.05$).

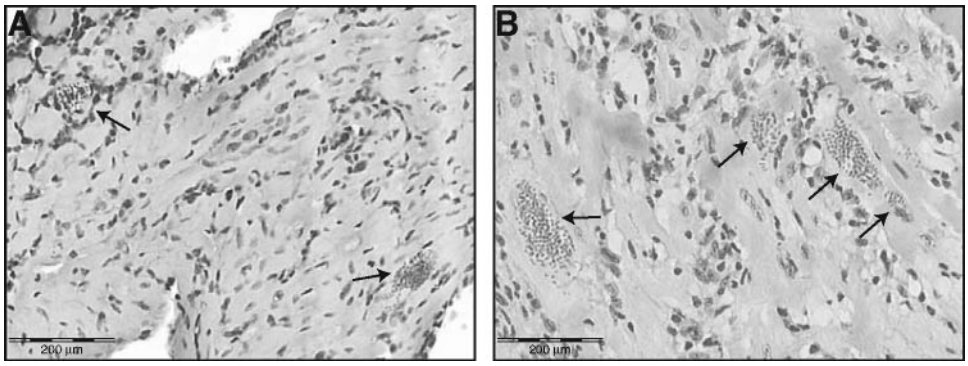

FIG. 3. Cardiac parasitism is increased in CCR5$^{-/-}$ mice at day 30 p.i. Heart tissue was fixed in formalin, paraffin embedded, cut into 5-μm sections, and stained with hematoxylin and eosin. Shown are representative sections from CCR5$^{+/+}$ mice (A) and CCR5$^{-/-}$ mice (B). Infected cells as indicated by arrows are generally larger and more numerous ($P \leq 0.01$, Mann-Whitney test) in CCR5$^{-/-}$ mice than in CCR5$^{+/+}$ mice.

(Fig. 2A). No further increase in mortality within *T. cruzi*-infected CCR5$^{-/-}$ mice was detected. Correlating with the increase in mortality in CCR5$^{-/-}$ mice was a significant increase in parasitemia between days 21 to 35 p.i. compared to CCR5$^{+/+}$ mice (Fig. 2B). Parasitemia peaked between 28 and 35 days p.i. in CCR5$^{-/-}$ mice; however, this was eventually controlled and no differences in parasite levels between CCR5$^{-/-}$ and CCR5$^{+/+}$ mice were detected past day 42 p.i. (Fig. 2B).

Parasite burden and inflammation within the heart. In addition to having higher parasitemia, CCR5$^{-/-}$ mice also have increased cardiac parasitism compared to CCR5$^{+/+}$ mice (Fig. 3 and Table 1). Detection of *T. cruzi*-infected cells within the hearts of both CCR5$^{+/+}$ and CCR5$^{-/-}$ mice is rare at day 15 p.i. with infected cells observed in only one mouse from each group of mice at this time point (Table 1). Cardiac parasitism peaks at day 30 p.i. in both groups of mice and is nearly five times higher in CCR5$^{-/-}$ mice, with an average of 163 infected cells/100 mm^2 of tissue compared to and average of 34 infected cells/100 mm^2 in CCR5$^{+/+}$ mice at this time point (Fig. 3 and Table 1). Cardiac parasitism is eventually controlled by day 60 p.i. in both groups with low numbers of infected cells present (Table 1). By day 120 p.i., cardiac parasitism is extremely rare in CCR5$^{-/-}$ mice, with only one infected cell observed out of six mice and was not observed in CCR5$^{+/+}$ mice (Table 1). There were no parasites detected in hearts of either CCR5$^{+/+}$ or CCR5$^{-/-}$ mice examined at day 200 p.i.

TABLE 1. Cardiac inflammation and tissue parasitism

Mouse strain	Time (days) p.i.	Avg inflammation score[a] ± SD		Tissue parasitism (mean no. of infected cells/ 100 mm^2 ± SE)	*n*
		Atrium	Ventricle		
CCR5$^{+/+}$	0	0.60 ± 0.55	0.67 ± 0.82	0.00	6
	15	2.11 ± 0.33	2.00 ± 0.00	0.18 ± 0.18	10
	30	4.00 ± 0.00	4.00 ± 0.00	34.20 ± 9.92	6
	60	3.75 ± 0.50	3.50 ± 0.58	1.40 ± .90	4
	120	2.25 ± 0.50	2.00 ± 0.00	0.00	4
	200	3.00 ± 0.00	3.67 ± 0.58	0.00	3
CCR5$^{-/-}$	0	0.40 ± 0.55	0.80 ± 0.45	0.00	5
	15	0.40 ± 0.55[b]	0.57 ± 0.53[b]	0.70 ± 0.70	7
	30	3.67 ± 0.65	3.67 ± 0.65	163.64 ± 25.97[b]	12
	60	3.00 ± 1.00	3.50 ± 0.58	2.34 ± 1.52	4
	120	3.50 ± 1.22	3.17 ± 0.75	0.29 ± 0.29	6
	200	2.25 ± 0.50	2.50 ± 0.58	0.00	4

[a] Using the numerical scoring system of Sun and Tarleton (43).
[b] Significant difference between CCR5$^{-/-}$ and CCR5$^{+/+}$ mice at the same time p.i., as determined by the Mann-Whitney test ($P \leq 0.01$).

The severity of inflammation within both the ventricle and atrium of infected mice was also evaluated (43). There are significantly fewer inflammatory cells in the atria and ventricles of CCR5$^{-/-}$ mice compared to CCR5$^{+/+}$ mice at day 15 p.i., suggesting that infiltration of mononuclear cells is delayed in CCR5$^{-/-}$ mice (Table 1). Analysis of cell surface markers expressed within infected tissues at this time revealed a significant decrease in transcripts associated with both CD4$^+$ and CD8$^+$ T cells, as well as macrophages (Fig. 4A and B). The reduced leukocyte accumulation in the heart that is observed at day 15 p.i. in CCR5$^{-/-}$ mice is eventually restored by day 30 p.i., from which point there is no significant difference in the degree of inflammation involving T cells and macrophages between wild-type and CCR5$^{-/-}$ mice out to 120 and 200 days p.i. (Fig. 4B and Table 1).

Expression of cytokines and chemokines in the heart. To evaluate the contribution of CCR5 signaling to cytokine and chemokine expression within the heart following *T. cruzi* infection, RPAs were used to quantify transcript levels at days 15, 30, 60, and 120 p.i. of CCR5$^{+/+}$ and CCR5$^{-/-}$ mice. The expression of inflammatory cytokines IFN-γ, TNF-α, and interleukin-1β (IL-1β) are significantly increased in the hearts of CCR5$^{-/-}$ mice at days 30 and 60 p.i. (Fig. 4C and D). The expression of IFN-γ and TNF-α is more than 1.5-fold higher and the expression of IL-1β is more than 3.5-fold higher in CCR5$^{-/-}$ mice than in CCR5$^{+/+}$ mice at day 30 p.i. At day 60 p.i., the expression of IFN-γ, TNF-α, and IL-1β are >2-fold higher in CCR5$^{-/-}$ than in CCR5$^{+/+}$ mice. The expression of these cytokines decreases to wild-type levels in CCR5$^{-/-}$ mice by day 120 p.i. (Fig. 4C and D). The level of chemokine expression in the heart was very similar between CCR5$^{+/+}$ and CCR5$^{-/-}$ mice at all time points except day 60 p.i. (Fig. 4D and E). At this time, the level of CXCL9 and CCL5 expression in the hearts of CCR5$^{-/-}$ mice was at least 1.5-fold higher than that observed in CCR5$^{+/+}$ mice.

Generation of *T. cruzi*-specific T cells. The delay in cardiac inflammation seen in CCR5$^{-/-}$ mice at day 15 p.i. suggests CCR5 enhances trafficking of lymphocytes and macrophages

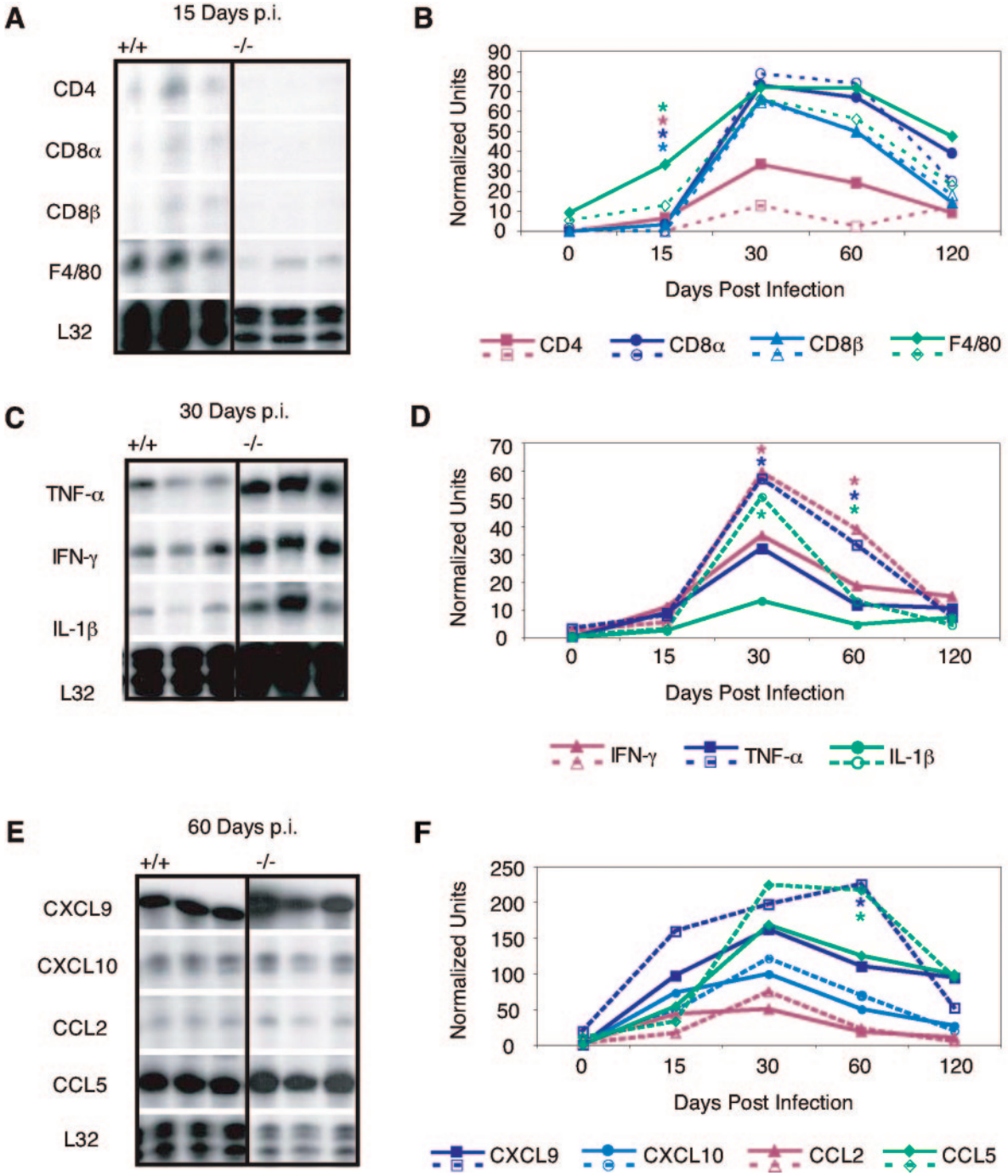

FIG. 4. Inflammatory cells and factors expressed within the hearts of $CCR5^{+/+}$ mice (solid lines) and $CCR5^{-/-}$ mice (dashed lines) were characterized by RPA at days 0, 15, 30, 60, and 120 p.i. Shown are representative RPA autoradiographs from three mice at the indicated times p.i. (A, C, and E) and the average quantitation of transcript expression normalized as a percentage of L32 expression (B, D, and F) for RPAs performed which assayed inflammatory cell marker expression (A and B), cytokine expression (C and D), and chemokine expression (E and F). An asterisk denotes significantly different from $CCR5^{+/+}$ expression as determined by using the Student *t* test ($P \leq 0.05$).

into the heart at early times after infection. However, it is also possible that T-cell activation is impaired or delayed in the absence of CCR5. To experimentally address the latter possibility, macrophage activation assays were performed with splenic T cells obtained from $CCR5^{+/+}$ and $CCR5^{-/-}$ mice at day 15 p.i. Briefly, T cells were purified from spleens of *T. cruzi*-infected $CCR5^{+/+}$ and $CCR5^{-/-}$ mice at day 15 p.i. and cultured with a macrophage cell line that had been incubated with HKT. IFN-γ produced by T cells in response to *T. cruzi* antigens presented by macrophages in culture was measured, as well as nitric oxide (NO) produced by macrophages in response to stimulation by T cells. $CCR5^{+/+}$ or $CCR5^{-/-}$ T cells

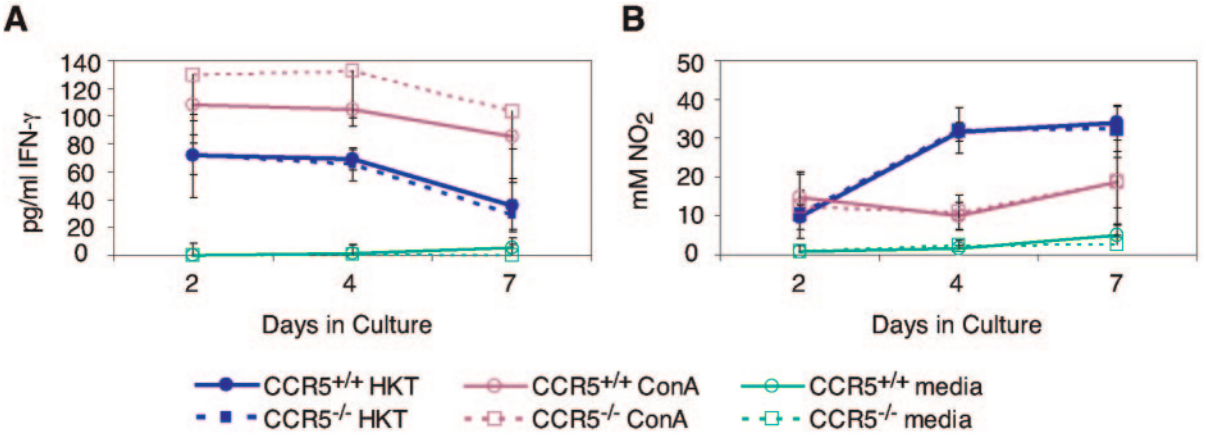

FIG. 5. CCR5$^{+/+}$ and CCR5$^{-/-}$ T cells secrete similar amounts of IFN-γ and stimulate production of similar levels of nitrite by IC-21 macrophages with cultured with HKT, ConA, or media. Splenic T cells were purified from CCR5$^{+/+}$ (solid lines) or CCR5$^{-/-}$ (dashed lines) T cells at day 15 p.i. and cultured with IC-21 macrophages plus HKT (blue), ConA (burgundy), or media (green). Culture supernatants were removed on days 2, 4, and 7 of culture; IFN-γ was measured by capture ELISA (A), and nitrite was measured by using the Greiss reagent (B). Each value shown represents the mean value from triplicate cultures ± the standard deviation.

cultured with macrophages and HKT produce similar amounts of IFN-γ (Fig. 5A). In addition, there was no difference in IFN-γ levels between ConA-stimulated CCR5$^{+/+}$ or CCR5$^{-/-}$ T cells. In addition, macrophages from these cultures produced similar amounts of NO, as measured by nitrite levels within the supernatant (Fig. 5B). These data indicate that no defect in the development of a *T. cruzi*-specific T-cell response is readily observed in mice lacking CCR5.

CCR5 and leukocyte trafficking into the heart. To assess the possibility that CCR5 plays a crucial role in the infiltration of mononuclear cells into the heart, a series of adoptive transfer experiments was performed (Fig. 6 and Table 2). In brief, CCR5$^{+/+}$ and CCR5$^{-/-}$ were infected with 50 bloodstream trypomastigotes, and spleens were removed at day 15 p.i. A single-cell suspension of splenocytes was prepared from each donor population and transferred by intravenous injection to RAG1$^{-/-}$ mice that had been infected with *T. cruzi* 15 days earlier. Hearts were removed from recipient RAG1$^{-/-}$ mice at day 30 p.i. (day 15 after transfer of splenocytes), and the severity of the parasite burden and inflammation in the heart was examined. A count of infected cells in the heart at day 30 p.i. indicated that RAG1$^{-/-}$ mice receiving CCR5$^{-/-}$ splenocytes have a significantly increased parasite burden than RAG1$^{-/-}$ mice receiving CCR5$^{+/+}$ splenocytes (Table 2). However, CCR5$^{-/-}$ splenocytes apparently provide some degree of protection to RAG1$^{-/-}$ mice because cardiac parasitism in RAG1$^{-/-}$ mice receiving CCR5$^{-/-}$ splenocytes is significantly lower than in control RAG1$^{-/-}$ mice receiving HBSS. The decreased parasitism in RAG1$^{-/-}$ mice receiving wild-type splenocytes coincides with a significant overall increase in cardiac inflammation relative to RAG1$^{-/-}$ receiving either CCR5$^{-/-}$ splenocytes or HBSS (Table 2). Inflammation observed in RAG1$^{-/-}$ mice receiving HBSS was composed of monocyte-derived macrophages as confirmed by RPA (data not shown). In addition, mortality was observed in RAG1$^{-/-}$ mice receiving wild-type and CCR5$^{-/-}$ splenocytes, 33 and 22%, respectively, whereas no mortality was observed in mice receiving only HBSS. The mortality observed is likely the result of T-cell and macrophage effector function (6), indicating that while infiltration of CCR5$^{-/-}$ splenocytes is reduced, effector function is retained, in agreement with expression of cytokines and chemokines in the hearts of CCR5$^{-/-}$ mice.

DISCUSSION

The principle finding derived from this study is that CCR5 enhances host defense during acute disease after infection of

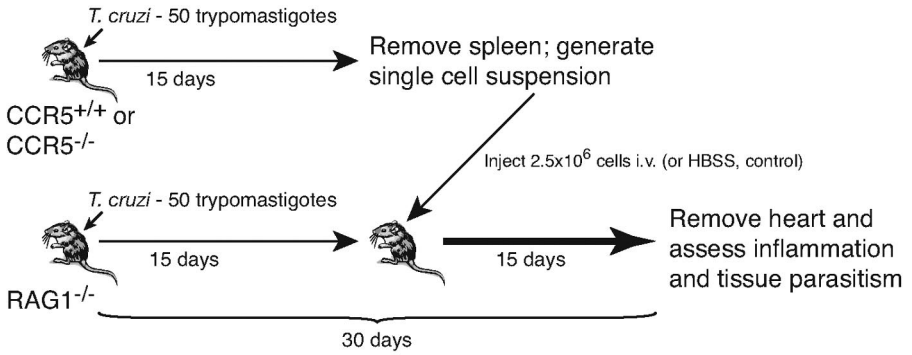

FIG. 6. Experimental design for adoptive transfer of CCR5$^{+/+}$ and CCR5$^{-/-}$ splenocytes to infected RAG1$^{-/-}$ mice.

TABLE 2. Cardiac inflammation and tissue parasitism in RAG1$^{-/-}$ mice[a]

Spleenocytes transferred	Time (days) p.i.	Avg inflammation score[a] ± SD		Tissue parasitism (avg no. of infected cells/100 mm^2 ± SE)	No. of survivors/ total no.
		Atrium	Ventricle		
CCR5$^{+/+}$	30	3.60 ± 0.55*§	2.67 ± 0.52*§	733.75 ± 327.38*§	6/9
CCR5$^{-/-}$	30	2.43 ± 0.79	2.14 ± 0.38	1,410.89 ± 485.54§§	7/9
HBSS	30	2.29 ± 0.49	2.00 ± 0.00	2,917.97 ± 664.51	8/8

[a] The average inflammation score is based on the numerical scoring system of Sun and Tarleton (43). Symbols: *, significantly different from mice receiving CCR5$^{-/-}$ spleenocytes (Mann-Whitney test, $P \leq 0.1$); §, significantly different from mice receiving HBSS (Mann-Whitney test, $P \leq 0.05$); §§, significantly different from mice receiving HBSS (Mann-Whitney test, $P \leq 0.1$).

susceptible mice with *T. cruzi*. Specifically, our results indicate that both parasitemia and cardiac parasitism are significantly higher when CCR5 signaling is ablated. In addition, our data support a role for CCR5 in regulating leukocyte inflammation within targeted tissues rather than influencing the generation of parasite-specific T cells. Finally, CCR5 is not essential for the maintenance of chronic inflammation within the heart. These studies support and extend recent work by others indicating that CCR5 expression correlates with optimal host defense by controlling parasite replication (25, 27, 38).

The findings that CCR5$^{-/-}$ mice have increased parasitemia days 21 to 35 p.i. and cardiac parasitism at day 30 p.i., during the acute stage of infection, may result from the fact that CCR5-deficient macrophages have reported defects in macrophage activation (16, 20, 39, 52). Activated macrophages amplify resistance to *T. cruzi* by generating reactive nitrogen intermediates that are toxic to *T. cruzi* and function to control parasite replication (12, 19, 48). Furthermore, signaling by CC chemokines (including the CCR5 ligands CCL3 and CCL5) increase trypanocidal activities of macrophages by enhancing their phagocytosis of *T. cruzi* and production of NO (2, 24, 49). Ultimately, CCR5$^{-/-}$ mice were able to control parasite replication during chronic disease within the blood by day 42 p.i. and heart by day 60 p.i. despite increased parasite burden during acute disease.

In addition to participating in macrophage activation, CCR5 could also contribute to host defense by regulating the generation of antigen-specific T cells and/or modulating inflammation. Previous studies investigating the role of CCR5 in control of *T. cruzi* infection during acute disease suggest that T-cell effector function may be compromised in the absence of CCR5, accounting for the increased parasite burden in CCR5$^{-/-}$ mice (25). However, the demonstration that T cells obtained from *T. cruzi*-infected CCR5$^{-/-}$ mice are capable of activating macrophages through secretion of IFN-γ in a comparable level with CCR5$^{+/+}$ T cells argues against a defect in T-cell activation in the absence of CCR5 signaling in this model. Indeed, examination of cytokine expression within the hearts of *T. cruzi*-infected mice revealed significantly higher transcript levels for both IFN-γ and TNF-α in CCR5$^{-/-}$ mice at days 30 and 60 p.i. that corresponds to the control of parasite replication and clearance of infected cells in the heart. Consistent with these findings are other studies that have reported similar increased production of cytokines, including IFN-γ, in CCR5$^{-/-}$ mice, suggesting that CCR5 signaling may influence cytokine secretion (14, 39). The results of the adoptive transfer experiments highlight an important role for CCR5 in promoting leukocyte infiltration into the heart and controlling parasite replication. These findings are also supported by the overall reduction in the severity of inflammation during acute disease observed in CCR5$^{-/-}$ mice compared to CCR5$^{+/+}$ mice. Furthermore, this is consistent with previous studies clearly demonstrating defects in T-cell and macrophage trafficking to sites of infection in CCR5-deficient mice (15, 16, 20). Importantly, our findings are in agreement with studies by Machado et al., in which infection of CCR5$^{-/-}$ mice with the more virulent Y strain of *T. cruzi* resulted in decreased lymphocyte trafficking to the heart during acute infection associated with decreased control of parasite replication (25). Our studies support and extend these findings by characterizing the role of CCR5 in chronic *T. cruzi* infection, as well as acute infection using the comparatively less virulent Colombiana strain, which produces disease in mice more similar to that observed in human infection (10, 44). Infection with the Y strain of *T. cruzi* results in 50% mortality of wild-type mice and 100% mortality of CCR5$^{-/-}$ mice by day 30 p.i. (25), whereas infection with the Colombiana strain of *T. cruzi* results in no mortality of wild-type mice and 20% mortality by day 60 in CCR5$^{-/-}$ mice, allowing for examination of chronic infection in CCR5-deficient mice. In addition, the prolonged functional analysis of the role of CCR5 in defense/disease out to 200 days p.i. provides important information with regard to how signaling through this receptor may modulate immune responses during chronic stages of disease.

During chronic *T cruzi* infection (day 60 to 200 p.i.), we found that the level of inflammation in CCR5$^{-/-}$ mice was equivalent to that observed in CCR5$^{+/+}$ mice despite the high level of expression of CCR5 and its ligand CCL5 during chronic infection. Thus, CCR5 does not play an essential role in maintaining inflammation in the heart during the chronic phase of *T. cruzi* infection. This suggests that while targeting chemokine/chemokine receptor interactions may provide therapeutic targets for treating some chronic inflammatory disease (7, 13, 30, 32), targeting CCR5 may not lead to a reduction of chronic inflammation in humans infected with *T. cruzi* and might not be an efficacious strategy for treating Chagas' disease.

Among the chemokines expressed within the heart of *T. cruzi*-infected mice are the CCR5 ligands CCL3, CCL4, and CCL5 (17, 25, 44). Recent studies have suggested an important role for CCL5 in promoting resistance to *T. cruzi* (25). However, treatment of *T. cruzi*-infected mice with anti-CCL5 neutralizing antibody did not directly affect parasite levels nor did it attenuate the inflammatory response within the heart (17), whereas treatment with Met-RANTES resulted in decreased infiltration of CD4$^+$ and CD8$^+$ T cells in the heart (27). This

suggests that other CCR5 ligands CCL3 and CCL4 may be functionally important in defense following parasite infection. More likely is the possibility of overlapping functional roles for chemokines in participating in defense during acute disease after *T. cruzi* infection, since our analysis of the expression of chemokine receptors in the heart show expression of CCR1 during acute infection and CCR2, CCR5, and CXCR3 during both acute and chronic infection. Importantly, the findings presented here indicate that CCR5 signaling is important in controlling parasite replication by (i) promoting inflammation within infected tissues and (ii) perhaps activating circulating monocytes and/or resident macrophages to produce NO. Further studies are needed to clarify the role of CCR5 in macrophage activation and trypanocidal activity. Collectively, these results demonstrate the importance of chemokines in regulating host defense in response to *T. cruzi* infection.

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