Modulation of Chemokine Production and Inflammatory Responses in Interferon-γ- and Tumor Necrosis Factor-R1-Deficient Mice during Trypanosoma cruzi Infection

Júlio C. S. Aliberti,* Janeusa T. Souto,* Ana P. M. P. Marino,‡ Joseli Lannes-Vieira,† Mauro M. Teixeira,‡ Joshua Farber,§ Ricardo T. Gazzinelli,‡ and João S. Silva*

From the Departments of Biochemistry and Immunology,* School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil; the Federal University of Minas Gerais,‡ Belo Horizonte, Brazil; the Department of Immunology, Fundação Oswaldo Cruz,§ Rio de Janeiro, Brazil; and the Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

Infection with Trypanosoma cruzi causes a strong inflammatory reaction at the inoculation site and, later, in the myocardium. The present study investigates the role of cytokines as modulators of T. cruzi-induced chemokine expression in vivo and in vitro. In macrophage cultures, although the stimulation with interferon (IFN)-γ increases the expression of IP-10, it blocks KC expression. Tumor necrosis factor (TNF)-α, on the other hand, potentiates KC, IP-10, macrophage inflammatory protein-1α, and JE/monocyte chemotactic protein-1 expression. Interleukin-10 and transforming growth factor-β inhibited almost all chemokines tested. The role of IFN-γ and TNF-α in chemokine modulation during infection was investigated in T. cruzi-infected IFN-γ-deficient (GKO) or TNF-R1/p55-deficient (p55−/−) mice. The expression of chemokines detected in the inoculation site correlated with the infiltrating cell type observed. Although GKO mice had a delayed and intense neutrophilic infiltrate correlating with the expression of KC and macrophage inflammatory protein-2, none of the above was observed in p55−/− mice. The detection of infiltrating T cells, Mφ, and IP-10 in the myocardium was observed in wild-type and p55−/−, but not in GKO mice. Together, these results suggest that the regulatory roles of IFN-γ and TNF-α on chemokine expression may play a crucial role in the modulation of the inflammatory response during T. cruzi infection and mediate resistance to infection. (Am J Pathol 2001, 158:1433–1440)

Trypanosoma cruzi is a hemoflagellate protozoan parasite that is the etiological agent of Chagas’ disease, a major public health problem in South and Central America. The infection is primarily characterized by an acute phase with detection of circulating parasites and an impressive inflammatory reaction at the site of parasite penetration. A few days later, significant myocarditis associated with tissue parasitism are noted and these are the most relevant pathological findings during infection with T. cruzi.

The resistance of inbred mice to acute infection with T. cruzi has been shown to be dependent on interferon (IFN)-γ that activates macrophages to produce nitric oxide (NO) and kill the obligate intracellular amastigote form of the parasite.1–6 In addition, tumor necrosis factor (TNF)-α provides a second signal stimulating NO production and anti-T. cruzi activity in IFN-γ-activated macrophages. Because T. cruzi-infected macrophages produce TNF-α, this cytokine seems to mediate its trypanocidal function via an autocrine pathway.7

On the other hand, the down-regulatory cytokines interleukin (IL)-10 and transforming growth factor (TGF)-β are associated with susceptibility to infection8–9 by inhibiting IFN-γ-mediated macrophage activation. Thus, neutralization of endogenous IL-10 leads to an increased T. cruzi-induced IFN-γ production and parasite killing.9,10 These results suggest that IL-10 may be a potent inhibitor of IFN-γ production during T. cruzi infection in mice and that the early resistance to infection is a result of the balance between IFN-γ and IL-10 produced.9

The mechanisms by which cytokines control parasite replication and myocarditis in T. cruzi-infected mice are still a matter of controversy. One distinct possibility is that cytokines modulate the expression of chemokines that in turn drive the inflammatory infiltrates observed during the acute and chronic phases of infection. In fact, T. cruzi infection induces the production of inflammatory and regulatory cytokines,7,8,11–13 and the β-chemokines macro-
phage inflammatory protein (MIP)-1α, MIP-1β, RANTES, and JE in macrophages14 or in cardiomyocytes.15 The importance of the production of these chemokines to disease outcome and host immunopathology during infection is not known.

In the present study, we evaluated the role of IL-10, TGF-β, IFN-γ, and TNF-α in regulating chemokine production and expression as well as leukocyte influx. We found that IFN-γ, TNF-α, IL-10, and TGF-α are indeed able to modulate the expression of various chemokines in T. cruzi-infected macrophages. Moreover, we found that the decreased inflammatory infiltrate in the peritoneum and heart of IFN-γ-deficient (GKO) and in TNF-R1-deficient (p55−/−) mice correlated with the altered expression of chemokines. These results suggest an important role for regulatory cytokines, especially IFN-γ and TNF-α, in the control of chemokine expression and production in experimental T. cruzi infection.

Materials and Methods

Experimental Animals

Five- to 6-week-old female C3H/HeJ, C57BL/6 (WT), or IFN-γ-deficient (GKO) or TNF-α receptor p55 (p55−/−) or p75 (p75−/−)-deficient mice were bred and maintained under standard conditions in the animal house of the Department of Immunology, University of São Paulo, Ribeirão Preto, Brazil. Deficient mice were obtained from The Jackson Laboratories (Bar Harbor, ME).

Parasites

The Y strain of T. cruzi was used in all experiments. For in vitro experiments, trypomastigotes were grown and purified from a monkey fibroblast cell line (LLC-MK2). For in vivo experiments, bloodstream trypomastigotes were obtained from infected animals. The levels of parasitemia were evaluated in 5 μl of blood drawn from the tail vein.

Differential Counting of Leukocytes and Detection of Chemokine mRNA Expression in Vivo

Mice were infected intraperitoneally with 1 × 10⁴ bloodstream trypomastigotes. At different times after infection peritoneal cavities were washed with cold phosphate-buffered saline (PBS). Total peritoneal host and parasite cells were counted in a hemocytometer; 1 × 10⁶ peritoneal cells were cytospun to slides and stained with May-Grünewald-Giemsa; leukocyte populations were determined after differential counting using standard morphological criteria.

FACS Analysis

For flow cytometry, 1 × 10⁶ cells were incubated in the presence of 5 μl of the fluorescein isothiocyanate-conjugated antibodies anti-CD3, anti-CD4, anti-CD8, and anti-CD22 (Pharimingen, San Diego, CA) for 30 minutes at 4°C in the dark according to the supplier’s specifications. The cells were then washed and resuspended in PBS-1% formaldehyde. Data acquisition was performed using a FACSorter, (Becton-Dickinson Immunocytometry System Inc., San Jose, CA). The percentage of each subset was used together with the total cell counting described above to calculate total numbers of each lymphocyte subset in the peritoneal exudate.

Macrophage Cultures for RNA Extraction

BALB/c mouse inflammatory macrophages were harvested from peritoneal cavities 3 days after infection of 1 ml of 3% sodium thioglycollate. The cells were washed and suspended to 1 × 10⁶ cells/ml in RPMI 1640 (Sigma), supplemented with 5% fetal bovine serum, 5 × 10⁻⁶ mol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, and antibiotics. The adherent cells were obtained after 2 to 4 hours incubation of single-cell suspensions in 24-well tissue-culture plates at 37°C. Nonadherent cells were removed and tryptomastigote forms were added at 1:1 parasite:cell ratio to macrophages and incubated for 6 hours at 37°C in a humidified chamber containing 5% CO₂. The cells were then washed three times and 0.5 ml of Trizol LS reagent was added to each well, incubated at room temperature for 5 minutes, and stored at −70°C until RNA extraction.

Total RNA Extraction and cDNA Preparation by Reverse Transcription

For reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, cells were collected by centrifugation of the peritoneal lavage fluid and RNA was prepared from the cell pellet. The extraction of total RNA was performed using 1 ml of the Trizol LS reagent according to the manufacturer’s recommendations (Life Technologies, Inc., Gaithersburg, MD). Each RNA sample was resuspended in water at 0.5 μg/μl. cDNA was synthesized from 2 μl of sample using Superscript II reverse transcriptase according to the supplier’s specifications (Life Technologies, Inc.).

Chemokine mRNA Detection

mRNAs for the C-X-C chemokines (Mig, IP-10, KC, MIP-2), the CC chemokines (MIP-1α, RANTES, JE/MCP-1), hydroxyphosphoribosyltransferase (HPRT), and β-actin were analyzed by RT-PCR. PCRs were performed using Taq polymerase (Life Technologies, Inc.) in a PTC-100 thermal cycler (MJ Research, Watertown, MA). The primer sequences and PCR product sizes for the genes above have been previously published14,16 and validate in the laboratory using plasmid containing the genes for each chemokine. Reaction conditions were 30 cycles of 1 minute at 94°C, 1 minute at 54°C, and 2 minutes at 72°C, with a final extension step of 7 minutes at 72°C. For each set of primers a negative sample (water) was run in
Disregulated Inflammatory Cell Infiltrates at the Inoculation Site of *T*. *cruzi* in IFN-γ- and TNF-RI-Deficient Mice

To evaluate the contribution of IFN-γ and TNF-R p55 and p75 in the response against *T*. *cruzi* infections, GKO, p55−/− and p75−/− mice were infected intraperitoneally with trypomastigote forms of the Y strain and parasitemia and mortality evaluated throughout the acute phase. As shown in Figure 2A, GKO presented an uncontrolled parasitemia that kept growing until mortality began. Similarly, p55−/− mice had a significantly higher parasitemia than either wild-type (WT) or p75−/− mice. The survival rates were found to be consistent with the levels of parasitemia observed (Figure 2B), ie, both GKO and p55−/− mice had an early mortality, which was not observed in WT and p75−/− mice. The above data suggest the critical role of IFN-γ and p55 in controlling parasite replication in vivo. Once these genes seemed to play an important role for resistance against *T*. *cruzi*, we questioned whether IFN-γ and TNF-RI could also play a role in the modulation of the inflammatory cell infiltrates in vivo. To achieve this goal the composition of inflammatory infiltrates in the site of inoculation was evaluated in WT, GKO, and p55−/− mice infected with trypomastigote forms of *T*. *cruzi*.

Kinetic experiments showed that neutrophils peaked early (24 hours) whereas lymphocytes peaked much later (day 8) after *T*. *cruzi* infection in WT mice (Figure 3, top, middle, and bottom). Two peaks of macrophage infiltration were noted on days 1 and 8. The neutrophil, macrophage, and lymphocyte infiltrate observed in infected GKO mice was late, peaking 9 days after infection, and it was more intense than that observed in WT animals (Figure 3, top, middle, and bottom). No neutrophil or macrophage infiltration was noted in p55−/− mice throughout the observation period. On the other hand, in p55−/− mice lymphocytes were raised as early as 2 days after infection and were persistently elevated throughout the observation period (Figure 3, bottom). The parasite counting at the inoculation site (peritoneal cavity, Table 1) showed a consistent correlation with the parasitemia levels (Figure 2A) and both GKO and p55−/− mice exhibited a significantly higher parasite replication than WT mice.

Results

IFN-γ, TNF-α, IL-10, and TGF-β Regulate the Expression of Chemokine mRNAs by *T*. *cruzi*-Infected Macrophages

Noninfected control macrophages only express little message for the chemokine RANTES (Figure 1A). Addition of TNF-α or IFN-γ increased message for RANTES and IP-10 and TNF-α also induced MIP-1α message (Figure 1A). IL-10 induced significant message for KC but none for the other chemokines, whereas TGF-β only blocked RANTES expression (Figure 1A). The infection of macrophages with *T*. *cruzi* induced significant message for MIP-1α, MIP-1β, KC, and IP-10, and enhanced the message for RANTES (Figure 1B). To test the effects of modulatory cytokines on the regulation of chemokines in vitro, macrophages were infected with *T*. *cruzi* together with modulatory cytokines. The addition of TNF-α enhanced the expression of MIP-1α, KC, and IP-10 and had no apparent effect on RANTES or MIP-1β expression (Figure 1B). The addition of IFN-γ to infected macrophages down-modulated KC and MIP-1α and enhanced RANTES and IP-10 expression (Figure 1B). Similarly to its effects on control macrophages, IL-10 increased KC expression in infected cells (Figure 1B). Interestingly, TGF-β decreased the expression of all chemokines tested in these experiments (Figure 1B).


document
Immunophenotyping of Infiltrating Lymphocytes at the Site of T. cruzi Infection in IFN-γ- and TNF-R1-Deficient Mice

The changes in lymphocyte subtypes at the site of inoculation during the course of T. cruzi infection in WT animals were mild with a tendency toward a decrease of CD22⁺ and increase of CD3⁺, CD4⁺, and CD8⁺ lymphocyte numbers in days 5 to 8 after infection. Overall, the lymphocyte subtypes in infected GKO mice were similar to those found in WT animals with a small but significant increase in CD22⁺ cells at day 8 after infection. The major variations of lymphocyte subtype infiltration were observed in p55⁻/⁻ mice. In these animals, there was an increase in the numbers of lymphocyte subtypes from day 1 to 8 of the infection, with CD3⁺, CD4⁺, and CD8⁺ lymphocytes peaking at day 8 and CD22⁺ lymphocytes peaking earlier after infection (Figure 4).

Chemokine mRNA Expression at the Site of Inoculation during T. cruzi Infection in IFN-γ and TNF-R1-Deficient Mice

The kinetics of in vivo mRNA expression of chemokines was evaluated in peritoneal exudate cells and is shown in Figure 5. In WT mice, there was significant expression of KC mRNA that was limited to the early days after infection and was not accompanied by the expression of MIP-2. In
GKO mice, both KC and MIP-2 mRNAs were expressed persistently in higher levels. No expression of KC or MIP-2 was observed in p55$^{-/-}$ mice. Significant levels of IP-10, Mig, and RANTES were detected in WT animals from day 3 after infection. In GKO animals no message for these chemokines was detected, whereas in p55$^{-/-}$ mice the chemokine message was detected in higher levels after 5 days of infection. Interestingly, only low levels of MIP1-$\alpha$ were detected in WT or p55$^{-/-}$ mice whereas mRNA for this chemokine was persistently elevated in infected GKO mice.

Detection of the C-X-C Chemokines Mig and IP-10 in Myocardium of T. cruzi-Infected Mice

The modulation of subpopulations of infiltrating lymphocytes and the expression of chemokines in the myocardium of GKO and p55$^{-/-}$ mice were evaluated by immunofluorescence. WT animals showed increased numbers of CD4- and CD8-positive cells in the myocardium on the ninth day after infection (Figure 6, a and b). In contrast, GKO mice did not show significant increases in the numbers of CD4- or CD8-positive cells (data not shown). On the other hand and in contrast to the increased number of lymphocytes at the site of inoculation, p55$^{-/-}$ mice showed a trend toward decreasing numbers of CD4- or CD8-positive cells when compared to WT animals, at least until the ninth day of the infection (Figure 6, c and d). Interestingly, GKO mice showed an increased number of F4/80$^+$ cells on the ninth day after infection, which was not observed in WT mice (data not shown). To establish a correlation between the modulation of lymphocyte migration and chemokine expression, serial sections of heart tissue from infected mice were immunostained for the chemokines IP-10 and Mig, well-known lymphocyte chemoattractants. Heart sections of WT animals (Figure 6, a and b) were found to be strongly positive for IP-10 and Mig on the ninth day after infection,
in agreement with the presence of CD4- and CD8-positive cells in the tissue. On the other hand, we did not detect production of Mig and IP-10 in GKO mice (data not shown), p55−/− mice showed a restricted pattern of Mig staining associated with modest perivascular infiltrates (Figure 6, c and d). To confirm these data we evaluated chemokine mRNA expression in the myocardium of these animals before and in the ninth day after infection. We found a strong infection-induced expression of the C-X-C chemokines Mig and IP-10 in the heart of infected WT mice (Figure 7). Confirming the immunohistochemical observations, GKO mice did not show detectable levels of mRNA for Mig and IP-10, whereas infected p55−/− mice did. Despite the complete absence of Mig and IP-10 expression and CD4 and CD8 cells in GKO mice, a significantly increased number of nests were found in the myocardium (25.0 ± 3.3 parasite nests per section in GKO mice versus 3.5 ± 1.1 and 7.2 ± 1.9 in WT and p55−/− mice, respectively).
likely responsible for the dramatic accumulation of polymor-
phonuclear neutrophils in the peritoneal cavity (Figure 3). These results suggest that IFN-γ is a potent inhibitor of
KC-mediated neutrophil migration in vivo.

On the other hand, the enhancing effects of TNF-α on
trypomastigote-mediated KC expression (Figure 1) were
consistent with the observations made during infection of
p55−/− mice. These animals lack TNF-α-driven KC pro-
duction and did not show expression of KC at any point
during infection. Moreover, no neutrophilic infiltrate was
found at the inoculation site or in the myocardium. It is
possible that, in the absence of TNF-α signaling via the
p55 receptor, reduced induction of KC occurred and this
led to a failure to recruit neutrophils. Together, these data
suggest that KC is a main factor that mediates neutrophil
migration in vivo during T. cruzi infection and that, at least
in this infection, TNF-R1 is essential for the production of
KC. In contrast, animals deficient in the p75 TNF-α rece-
ceptor did not differ from WT mice in respect to leukocyte
recruitment or infection levels, suggesting that signaling
via the latter receptor does not play a role in acute T. cruzi
infection.

Lymphocyte recruitment in vivo was found to be signif-
ically enhanced in p55−/− animals. Interestingly, these
animals had lower expression of almost all chemokines
so far tested (Figure 5), with the exception of Mig. The
accumulation of lymphocytes in the peritoneal cavity of
p55−/− animals was predominantly composed of CD3⁺,
CD8⁺, and CD22⁺ cells on day 1 and CD3⁺, CD4⁺, and
CD8⁺ cells on day 7 after infection (Figure 4). These
results suggest that TNF-α down-regulates the recruit-
ment of lymphocytes to the site of infection via activation
of the TNF-R1 receptor. A possible beneficial conse-
quence of a limited recruitment of lymphocytes might be
to decrease the number of autoreactive cells within the
infiltrates. This is in agreement with the increased number
of inflammatory cells found in the late phase of T. cruzi
infection in the heart of p55−/− mice.20 On the other
hand, a possible negative consequence would be the
decrease of the number of parasite-specific cells, which
might contribute to the less efficient response to infection.

Our findings also suggest that, in the absence of an
adequate TNF-R1 signaling, Mig seems to be the main
mediator of lymphocyte migration in vivo during T. cruzi
infection. However, although GKO mice did not express
mRNA for the chemokines Mig, IP-10, and RANTES,
these animals showed intense lymphocyte accumulation
intrapertioneally, suggesting that other chemokines, such
as MIP-1α and JE/MCP-1 might be involved in lympho-
cyte recruitment to the latter site (Figures 3 and 4).

The direct correlation found between the presence of
CD4- and CD8-positive cells (Figure 6) in the myocar-
dium and the production of the chemokines Mig and
IP-10 in this tissue suggests that these chemokines may
be involved in the attraction of lymphocyte populations
to the hearts of infected mice. Moreover, the limited lympho-
cytic infiltrates in GKO and p55−/− mice, concomitantly
with the complete absence of Mig and IP-10 expression
and production in the heart of GKO and low levels in
p55−/− mice, strengthen the correlation. This could also
be observed during chronic chagasic myocarditis, in
which the expression of Mig and IP-10 remained high
concomitantly to an intense expression of IFN-γ mRNA.21

Considering that Mig and IP-10 are able to induce the
preferential migration of IFN-γ-producing cells by signal-
ing via CXCR3, the above results raise the possibility
that the cytokines IFN-γ and TNF-α may contribute to the
genesis of myocarditis through induction of Mig and IP-
10, which in turn, lead to the accumulation of IFN-γ-
producing T cells as a part of a positive feedback loop.

To provide further support for this observation, recently
published data showed that IP-10 is essential for the
accumulation of effector T cells that are required for
resistance to infection with Toxoplasma gondii.24

In GKO mice, not only did we observe a greater num-
ber of parasite nests but also a large increase in the size
of each nest (data not shown). This is in marked agree-
ment with our recent observation showing that cardiomy-
cytes controlled the replication of parasites in the pres-
ence of IFN-γ or TNF-α in a NO-dependent manner.15 Of
interest, we also showed that T. cruzi-infected cardiomy-
cytes could produce a significant amount of lympho-
cyte-active chemokines. These results raise the interest-
ing possibility that the production of chemokines by
cardiomyocytes could, in turn, increase the infiltration of
inflammatory cells in the heart and, then, control the
infection via the production of IFN-γ-driven NO produc-
tion. On the other hand, this cardiomyocyte/lymphocyte
cross talk could also be a relevant interaction explaining
the major pathological alterations observed in chronic
chagasic heart disease.

The role of chemokines and their receptors in modu-
inating innate as well as acquired immunity may not remain
only in driving cell trafficking to target organs and tissues.
Recent observations also point to direct effects of che-
mones in the activation of key cell populations for innate
and acquired immunity.25 The results presented herein
contribute to our understanding of the genesis and mod-
ulation of inflammatory reactions in T. cruzi-infected mice
both at the inoculation site and in the myocardium. Inas-
much as inflammation seems to play a major role in the pathophysiology of Chagas heart disease, our results suggest that antagonizing the action of chemokines, especially IP-10 and Mig, might be of benefit in the treatment of chagasic myocarditis.

Acknowledgments

We thank Dragana Jankovic for critical reading and suggestions, and Alexandra R. V. Dias for excellent technical assistance in FACS analysis.

References

24. Kahn IA, MacLean JA, Lee FS, Casciotti L, DeHaan E, Schwartzman JD, Luster A: IP-10 is critical for effector T cell trafficking and host survival in Toxoplasma gondii infection. Immunity 2000, 12:483–494