Monocytes from Patients with Indeterminate and Cardiac Forms of Chagas’ Disease Display Distinct Phenotypic and Functional Characteristics Associated with Morbidity

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Many studies have demonstrated that monocyte-derived macrophages display critical activities in immunity to parasites. The ability of these cells to process and present antigens, produce cytokines, and provide costimulatory signals demonstrates their pivotal role in initiating immune responses. Although potential modulatory function has been attributed to monocytes from patients with Chagas’ disease, a systematic phenotypic and functional analysis of these cells has not been performed. In this work, we analyzed the ex vivo expression of important surface molecules (CD11b and HLA-DR) and immunoregulatory cytokines (interleukin-10 [IL-10], IL-12 and tumor necrosis factor alpha [TNF-α]) in CD14+ and CD14− monocytes from Chagas’ disease patients with polar clinical forms of the disease: indeterminate or severe cardiac. We also evaluated the influence of in vitro infection with T. cruzi in the expression of such molecules. We observed that monocytes from indeterminate-disease patients display lower levels of HLA-DR than those from noninfected individuals both ex vivo and after in vitro infection with T. cruzi. Although ex vivo expression of CD11b was similar among the groups, in vitro infection led to decreased expression of this molecule by monocytes from Chagas’ disease patients but not from noninfected individuals. Analysis of the expression of immunoregulatory cytokines showed that while monocytes from indeterminate-disease patients are committed to IL-10 expression, a higher percentage of monocytes from cardiac-disease patients express TNF-α after exposure to live parasites. These results suggest that monocytes from indeterminate-disease patients display modulatory characteristics related to low HLA-DR and high IL-10 expression whereas monocytes from cardiac-disease, patients may be committed to induction of inflammatory responses related to high TNF-α expression.

Induction of efficient cellular immune responses is directly dependent on appropriate activation of antigen-presenting cells (APC). T-cell stimulation requires interaction between the T-cell receptor and the major histocompatibility complex (MHC)-peptide complex, as well as adequate costimulation provided by the APC. In contrast, it is clear that APC are often involved in down regulating T-cell responses. A strategy used by several pathogenic microorganisms to escape the host immune system is to down modulate the antigen-presenting and costimulatory functions of APC. As an example, it has been shown that several viruses express interleukin-10 (IL-10)-like molecules, which can suppress antigen presentation and other cellular functions (18). Similarly, parasites can induce the production of cytokines that induce a decrease in the expression of molecules critical for APC function and T-cell stimulation, such as MHC class II and costimulatory molecules (32).

Trypanosoma cruzi is an intracellular parasite that causes Chagas’ disease, an illness that leads to heart failure in 20 to 30% of infected individuals (27). Although Chagas’ disease is prevalent mostly in Latin America, where it affects millions of people, a considerable number of cases have been reported in North America (27). Despite the importance of the cardiac clinical form, most patients infected with T. cruzi have an asymptomatic form of the disease, named indeterminate, and a small percentage of patients with Chagas’ disease may develop the digestive clinical disease. The factors that determine the distinct clinical outcomes, leading to a mild or to a severe form, are not completely understood. However, it is clear that chagasic pathology is associated with the host immune response (reviewed in reference 4).

T cells play an important role in the dynamics of Chagas’ disease. We have previously shown that Chagas’ disease patients display high percentages of circulating activated HLA-DR+ T cells, as well as CD28− T cells (14, 15). Accordingly, activated T cells are the predominant cell type in the cardiac inflammatory lesions (29). While the presence of mRNA for inflammatory and anti-inflammatory cytokines was found in peripheral blood cells of patients with indeterminate and cardiac clinical forms (13), inflammatory cytokines such as tumor necrosis factor alpha TNF-α and gamma interferon were predominant at lesion sites (9, 29). Many studies have attempted to determine the stimuli responsible for T-cell activation in human Chagas’ disease. Parasite-derived antigens are able to stimulate CD4+ and CD8+ T cells from Chagas’ disease pa-
tients in vitro (11). Moreover, T cells that can recognize and proliferate in response to autologous antigens have also been detected in Chagas’ disease patients (8, 17). Interestingly, the removal of adherent cells from cultures of peripheral blood mononuclear cells (PBMC) from Chagas’ disease patients that respond with low intensity to parasite antigens (named “low responders”) led to an increase in the proliferative capacity of these cells (23), demonstrating the regulatory potential of APC over T-cell reactivity in chagasic patients.

Although T-cell responses seem to be critical in Chagas’ disease and are directly influenced by the APC, a detailed analysis of phenotypic and functional characteristics of monocytes from Chagas’ disease patients had not been performed to date. Considering clinical evolution and characteristics of the infection, we hypothesize that monocytes from indeterminate-disease patients display anti-inflammatory characteristics, as opposed to monocytes from cardiac-disease patients. To test this hypothesis, we evaluated the ex vivo expression of surface molecules involved with T-cell activation (HLA-DR and CD11b) and key immunoregulatory cytokines (IL-10, IL-12 and TNF-α) by monocytes from both patient groups. Furthermore, we exposed these cells to parasite antigens or subjected them to parasite infection in vitro and determined the effect of each treatment on the expression of the surface molecules and cytokines. Indeed, we observed that monocytes from indeterminate-disease patients display lower levels of HLA-DR and higher levels of IL-10 whereas monocytes from cardiac-disease patients display higher levels of TNF-α both ex vivo and after in vitro infection with live parasites. The characteristics of monocytes from indeterminate-disease patients are consistent with the establishment of a modulatory response, whereas monocytes from cardiac-disease patients may elicit T-cell activation in the presence of high levels of TNF-α, leading to exacerbated inflammation. Thus, our data demonstrate that monocytes from patients with different clinical forms of Chagas’ disease display distinct phenotypic and functional profiles. Specifically, the monocytes react differently on in vitro infection, suggesting possible mechanisms for control of T-cell responses and influencing their role in the development of pathology.

MATERIALS AND METHODS

Patients. Chagas’ disease patients included in our studies were from areas of endemic infection within the state of Minas Gerais, Brazil, and were under the medical responsibility of one of us (M.O.C.R.). Serologic tests indicative of Chagas’ disease were positive in all patients studied. The patients were in the chronic stage of the infection and had well-defined clinical forms classified as indeterminate or cardiac based on clinical, radiological, electrocardiographic, and echocardiographic analysis. Indeterminate-disease patients (n = 9) did not present with any clinical manifestations or alterations on clinical, radiological, and echocardiographic examination. Patients with dilated cardiopathy (n = 12) were classified as having the cardiac form of the disease and presented with severe heart disease, reflective of alterations in impulse generation and conduc-

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**RESULTS**

Ex vivo analysis of the expression of CD11b and HLA-DR in monocytes: indeterminate-disease, but not cardiac-disease, patients display lower levels of HLA-DR in CD14+ monocytes than do noninfected individuals. To determine whether there were any differences in the expression of molecules that were biologically relevant for monocyte function, such as antigen presentation and T-cell contact, we evaluated the expression of CD11b and HLA-DR by CD14+ cells from indeterminate-disease and cardiac-disease patients or noninfected individuals. Unstimulated monocytes were subjected to double labeling for the CD14 monocyte marker and CD11b or HLA-DR surface molecules. Gating on the monocyte region, we analyzed the total frequency of CD14+ monocytes as well as the intensity of expression of each molecule (Table 1). How-
cultures displayed a similar ability to infect cells (Table 2). When we compared the percentage of infection among CD11b<sup>+</sup> monocytes and CD3<sup>+</sup> and CD19<sup>+</sup> cells, we observed that the CD11b<sup>+</sup> monocytes were preferentially infected by trypomastigotes in all groups analyzed (Fig. 1A to C; Table 2). Approximately 1% of T cells and 5% of B cells present in the cultures were infected; this contrasted with a monocyte infectivity of approximately 80%.

We further measured the intensity of fluorescence expressed by CFSE-labeled parasites in each cell population to determine if the number of parasites per cell was also similar among the groups. Although the percentage of infected cells was similar, the intensity of infection was lower in monocytes from cardiac-disease patients than in monocytes from noninfected individuals (p < 0.05 [Fig. 1D; Table 2]). We did not observe any statistically significant differences in fluorescence intensity when comparing indeterminate-disease and cardiac-disease patients or indeterminate-disease patients and noninfected individuals. Moreover, the intensity of infection was similar in T and B cells from individuals from all groups (Fig. 1D; Table 2).

Thus, analysis of infection of adherent cells showed that while the percentage of infected cells was similar among the groups, the intensity of infection was lower in monocytes from cardiac-disease patients than in monocytes from noninfected individuals.

**Evaluation of the effects of T. cruzi infection on the expression of CD11b and HLA-DR by monocytes.** We evaluated the effect of in vitro infection with T. cruzi on the expression of molecules that play a major role in monocyte function. Infected adherent cells were submitted to double-label staining for the CD14 monocyte marker and CD11b or HLA-DR surface molecules. Gating on the monocytes, the frequency of expression of each molecule by the CD14<sup>-</sup> monocyte population was determined, as well as the intensity of expression of each molecule, as described for the ex vivo analysis in Materials and Methods.

Two different approaches were taken to analyze the data obtained: (i) comparison between cells infected with T. cruzi, cells exposed to T. cruzi antigens, and cells with no treatment within the same patient group; and (ii) comparison of each
treatment among indeterminate-disease and cardiac-disease patients and noninfected individuals. Exposure to *T. cruzi* antigens was used to determine whether infection, or simply exposure to parasite antigens, would be responsible for any observed changes.

Our results showed that CD11b expression by CD14+/H11001 monocytes was significantly decreased after *T. cruzi* infection of cells from indeterminate-disease and cardiac-disease patients, but not from noninfected individuals, compared to the respective noninfected control cells (Fig. 2A). Exposure to parasite antigen did not have any statistically significant effect on the expression of CD11b in any of the analyzed groups (Fig. 2A). We did not observe statistically significant differences when analyzing the mean intensity of expression of CD11b in double-positive cells as well as mean intensity of expression of each molecule within CD14+ cells. Identical symbols above the bars indicate statistical significance using the Tukey-Kramer test. Error bars indicate standard deviation.

We observed a lower frequency of HLA-DR expression in CD14+/H11001 cells from indeterminate-disease patients after in vitro infection with *T. cruzi* compared to those from the noninfected individuals, this difference was not statistically significant compared to the decreased expression of HLA-DR already seen in the ex vivo analysis of cells from indeterminate-disease patients. (Fig. 2A; Table 1). No phenotypic changes were observed within the CD14+ monocyte subpopulation on in vitro infection with *T. cruzi* or exposure to parasite antigen (data not shown).

Functional analysis of monocytes from Chagas' disease patients and noninfected individuals after in vitro infection with *T. cruzi*: different cytokine profiles in patients with distinct clinical outcomes. To determine whether in vitro infection with

<table>
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<tr>
<th>Source of <em>T. cruzi</em></th>
<th>% of CFSE +/H11001 cells</th>
<th>CD11b cells</th>
<th>CD3 cells</th>
<th>CD19 cells</th>
<th>CD11b cells</th>
<th>CD3 cells</th>
<th>CD19 cells</th>
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<tr>
<td>L929 cells</td>
<td>78.20 ± 86.11</td>
<td>71 ± 4.1</td>
<td>14 ± 0.5</td>
<td>11 ± 0.10</td>
<td>3 ± 0.07</td>
<td>1 ± 0.04</td>
<td>56 ± 3.5</td>
</tr>
<tr>
<td>Vero cells</td>
<td>61 ± 39</td>
<td>8 ± 0.6</td>
<td>53 ± 0.3</td>
<td>4.5 ± 0.5</td>
<td>4 ± 0.3</td>
<td>0.5 ± 0.1</td>
<td>7 ± 0.2</td>
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*Statistically significant, using Student's *t*-test (*P* < 0.05), compared to noninfected group.*
We observed that nonstimulated CD14+ cytokine expression by the monocytes from patients in the infection on the cytokine profile, we determined the ex vivo expression of IL-10, IL-12, and TNF-α by these cells via flow cytometry. Cells were stained with anti-CD14 monoclonal antibodies in conjunction with differentially labeled anticytokine antibodies, as described in Materials and Methods. Before analyzing the influence of in vitro infection on the cytokine profile, we determined the ex vivo cytokine expression by the monocytes from patients in the different groups, analyzing cells from nonstimulated cultures. We observed that nonstimulated CD14+ cells from Chagas’ disease patients, despite the clinical form, displayed a higher percentage of IL-10 and IL-12 expression than did nonstimulated cells from noninfected individuals (Fig. 3A and B, black bars). Expression of TNF-α was statistically similar when nonstimulated cells from individuals in the different groups were compared (Fig. 3C, black bars). Evaluating the effect of in vitro infection with T. cruzi on monocytes from patients in different groups, we observed that T. cruzi infection led to an increase in IL-10 and IL-12 expression by monocytes from indeterminate disease patients compared to that by cells cultured with medium only (Fig. 3A and B, compare cross-hatched and black bars). Although in vitro infection with T. cruzi seemed to induce an increase in IL-10 and IL-12 expression by CD14+ cells from cardiac-disease patients, these tendencies were not statistically significant compared to the medium control (Fig. 3A and B, compare cross-hatched and black bars). Infection with T. cruzi had no effect in IL-10, IL-12, or TNF-α expression by CD14+ monocytes from noninfected individuals (Fig. 3, compare cross-hatched and black bars). Interestingly, TNF-α expression was increased statistically significantly only in cells from cardiac-disease patients after in vitro infection with parasite (Fig. 3C, compare cross-hatched and black bars). The increase in the expression of TNF-α by monocytes from cardiac-disease patients and of IL-10 and IL-12 by monocytes from indeterminate-disease patients was dependent on infection, since the frequencies were not significantly altered on antigenic stimulation (Fig. 3). In fact, antigenic stimulation did not significantly change IL-10 expression by monocytes from individuals in any group. Although no significant changes were detected in IL-10, IL-12, and TNF-α expression by monocytes from noninfected individuals after infection with T. cruzi, antigenic stimulation led to a statistically significant increase in the frequency of IL-12 expression by CD14+ monocytes from noninfected patients (Fig. 3B, compare white and black bars). Thus, our data show that in vitro infection with T. cruzi induces differential cytokine expression by CD14+ cells from Chagas’ disease patients, with distinct clinical outcomes. Although it increases IL-10 and IL-12 expression by cells from indeterminate-disease patients, it induces a higher frequency of TNF-α expression in cells from cardiac-disease patients. Analyzing the ratio of IL-10 to TNF-α in indeterminate-disease and cardiac-disease patients after in vitro infection with T. cruzi gives values of approximately 0.8 for cardiac-disease patients and approximately 5 for indeterminate-disease patients, emphasizing a low expression of TNF-α and a high expression of IL-10 by CD14+ cells from indeterminate-disease patients. To determine whether T. cruzi infection would lead to functional changes in CD14+ monocytes, we also evaluated the expression of the cytokines in this cell subpopulation. We observed that T. cruzi infection led to an increase in TNF-α expression by CD14+ monocytes from indeterminate-disease and cardiac-disease patients but did not significantly alter IL-10 or IL-2 expression by CD14+ cells compared to the results for noninfected individuals (Table 3). Similar to the observation with CD14+ cells, the increase of TNF-α expression by CD14+ cells was also infection dependent in cells from indeterminate-disease and cardiac-disease patients, since antigenic exposure did not change the expression of this cytokine.
within CD14⁺ monocytes. Thus, our results showed that in vitro infection with T. cruzi induces changes in cytokine expression by monocytes from Chagas’ disease patients and that the observed changes are consistent with the clinical differences between patients with the indeterminate and cardiac forms of disease.

DISCUSSION

Many studies have demonstrated that monocyte-derived macrophages display critical activities in immunity to parasites. The encounter of differentiated monocytes with parasites is an essential step in the initial control of infections (reviewed in reference 30). Moreover, the ability of cells from the mononuclear phagocytic system (36). In this work, we demonstrated that circulating monocytes from Chagas’ disease patients display phenotypic differences from those from noninfected individuals. Moreover, we showed that stimulation of these cells, through in vitro infection with T. cruzi, reveals differential functional potentials of monocytes from Chagas’ disease patients with different clinical outcomes, suggesting a role for these cells in the outcome of pathology.

The influence of contact with live T. cruzi and/or infection by this parasite was previously analyzed using PBMC and monocytic cell lines from noninfected individuals (19, 33). It was shown that coculture of T. cruzi with human PBMC from noninfected individuals leads to lymphocyte proliferation, monocyte activation, and increased production of IL-1, IL-2, IL-5, IL-6, gamma interferon, and TNF-α (33). However, since whole PBMC were used in the absence of specific monocyte staining, the overall characteristics of the cultures were probably reflecting lymphocyte changes, since these cells predominated in the cultures, as suggested by the authors (33). Another study using monocyte and macrophage hydribomas determined that infection with T. cruzi leads to a decrease in HLA-DR expression on a cell-per-cell basis and an increase in the expression of the integrin LFA-1 (19). Moreover, the authors showed that while TNF-α and IL-6 expression was increased on infection, IL-1 levels were decreased. Thus, these early studies have suggested that the parasite can indeed cause changes in infected cells. Other studies have suggested that APC play a critical role in the modulation and induction of T-cell responses in cells from Chagas’ disease patients or in noninfected individuals that are exposed to parasite antigens (23, 26). Taken together, these findings raised the question whether qualitative differences existed in monocytes from Chagas’ disease patients and noninfected individuals and in monocytes from patients with different clinical forms of Chagas’ disease.

We analyzed the expression of CD11b and HLA-DR in CD14⁺ and CD14⁺ monocytes with different polar clinical forms from Chagas’ disease patients and from noninfected individuals prior to in vitro stimulation in order to evaluate the occurrence of alterations in the expression of these molecules in an ex vivo context. CD11b (Mac-1) is an integrin expressed by monocytes and is involved with cell interaction and recruitment (22). We observed that the expression of CD11b in CD14⁺ and CD14⁺ monocytes from individuals in the different groups was not significantly different. However, analysis of HLA-DR expression showed that indeterminate-disease patients, but not cardiac-disease patients, have lower expression of this molecule on CD14⁺ cells than do noninfected individuals. Since MHC class II molecules are essential for antigen presentation to CD4⁺ T cells, these data may indicate that indeterminate-disease patients display a lower ability to stimulate CD4⁺ T cells. This hypothesis could represent an important mechanism of controlling inflammatory reaction in indeterminate patients, where a lower inflammatory response would lead to less tissue damage, which is consistent with this form of the disease. We have previously observed a slightly lower proliferative response of CD4⁺ T cells from indeterminate-disease patients, compared to cardiac-disease patients, on stimulation with parasite antigens (11). Although those differences were not statistically significant, they may be indicative of lower antigenic presentation to CD4⁺ T cells in indeterminate-disease patients as a result of lower HLA-DR expression. Further analysis of these cells, directly addressing their ability to present antigens and control inflammation, would clarify this point.

Evaluation of infectivity of adherent cells from Chagas’ disease patients and noninfected individuals indicated that T. cruzi trypomastigotes of the Y strain are equally capable of infecting cells from individuals of all groups. We used parasites from Vero or L929 cells, since these cells are often used as hosts for obtaining T. cruzi trypomastigotes, and observed that the parasites had similar infectivity. Thus, the cell source did not influence the infectivity. Previous studies have suggested a role for Mac-1 (CD11b) in the infection of cells by trypano-
matides and indicated that the parasites that adhere to the cells are actually internalized, showing a direct correlation between adherence to cell surface and infection (28, 31). It is possible that CD11b may also be involved in the infection by the trypanosomatide T. cruzi and that the fluorescence observed using CFSE staining, due to either adherence or actual internalization of the parasite, is reflective of current or imminent infection. The fact that we did not observe differences in CD11b expression in monocytes from individuals in different groups could explain, in part, the similarity of infectivity. However, CD11b may not be the only ligand used by T. cruzi to enter cells. Recent studies have shown that a trans-sialidase named gp83-TSA is used by the parasite to attach to cells (35). Interestingly, although the percentage of infected cells was similar among groups, the intensity of infection was lower, on a cell-per-cell basis, in monocytes from cardiac-disease patients than in monocytes from individuals in the control group, as reflected by the lower intensity of CFSE (Fig. 1). It is possible that monocytes from cardiac-disease patients have a lower phagocytic ability than those from noninfected individuals or that they express smaller numbers of molecules involved with T. cruzi infection, other than CD11b, such as gp83-TSA ligand. Interestingly, serum from patients with cardiac disease decreased the infection of Vero cells by T. cruzi trypomastigotes (24). Thus, it is also possible that some soluble molecule present in cardiac-disease patients is inhibiting infectivity. Although T. cruzi trypomastigotes have a preference for infecting monocytes over other blood cells, their ability to infect lymphocytes has been shown (34). Our results corroborate these findings, showing that a very small percentage of T and B cells present among the adherent cells were infected by T. cruzi. Since our analyses were done after short-term cultures, we could not determine the importance of apoptosis in this system, as previously described in experimental models (16). However, studies evaluating this mechanism in cells from indeterminate-disease and cardiac-disease patients are under way in our laboratory.

In vitro infection with T. cruzi led to a decrease in expression of CD11b in monocytes from Chagas’ disease patients, which could represent a mechanism triggered by the parasite leading to less effective T-cell activation. This is supported by previous studies demonstrating the importance of CD11b in mediating macrophage–T-cell interactions (20). The fact that infection with T. cruzi did not alter CD11b expression in monocytes from noninfected individuals could reflect distinct behavior of monocytes from patients and noninfected individuals as a result of exposure to different in vivo cytokine environments. Supporting this idea, we have previously shown that Chagas’ disease patients display different cytokine profiles from those of noninfected individuals (13).

Cytokine expression by monocytes from Chagas’ disease patients and noninfected individuals was determined before and after in vitro infection with T. cruzi or exposure to parasite antigens. We observed that in vitro infection led to an increase in the expression of TNF-α by CD14+ monocytes from Chagas’ disease patients compared to monocytes from noninfected individuals. Interestingly, the expression of cytokines by CD14+ monocytes was much lower than that observed in CD14+ monocytes, suggesting that these cell populations are indeed functionally distinct. Our results showed that monocytes from Chagas’ disease patients display a higher percentage of expression of IL-10 and IL-12 than do cells from noninfected individuals. Moreover, in vitro infection with T. cruzi led to an increased expression of TNF-α in cells from cardiac-disease but not indeterminate-disease patients whereas high IL-10 levels were maintained in cells from indeterminate-disease but not cardiac-disease patients. The higher levels of IL-10 in cells from indeterminate-disease patients could explain the lower HLA-DR expression that we found in monocytes from these patients. Moreover, these results suggest that interactions of the parasite with cells from cardiac-disease patients induce a cascade of reactions that cause the production of inflammatory cytokines, whereas in cells from indeterminate-disease patients the balance is tipped toward the production of anti-inflammatory cytokines.

Studies using experimental models of T. cruzi infection have shown that glycosylphosphatidylinositol mucins induce the production of inflammatory cytokines by monocytes in vitro and in vivo (1, 2, 7, 10). Additionally, infection of a human monocyte hybridoma with T. cruzi leads to increased expression of inflammatory cytokines (19) whereas stimulation with glycosylinositolphospholipids induces a lower production of IL-10, IL-12, and TNF-α by activated human monocytes (5). Thus, whereas distinct parasite components may have differential stimulatory abilities on cells from patients, it is possible that the differences observed in our results are related to the microenvironment established in vivo in the patients with indeterminate or cardiac disease. Whether these differences are a cause or a consequence of the disease is a difficult question to answer; however, understanding the functions of monocytes and their in vivo potential provides important information for understanding the complex mechanisms involved in the differential clinical evolution of human Chagas’ disease.

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