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Insufficient TLR Activation Contributes to the Slow Development of CD8+ T Cell Responses in Trypanosoma cruzi Infection

Angel M. Padilla,* Laura J. Simpson,* and Rick L. Tarleton2*†

During experimental infection with Trypanosoma cruzi, mice develop a strong CD8+ T cell response focused mainly on a few immunodominant peptides encoded in trans-sialidase family genes. Despite the potency of this response, the initial emergence and peak of parasite-specific CD8+ T cells has been noted to be relatively slow. In this study, we further document this delayed onset of T cell responses to T. cruzi as measured by the increase in frequency of parasite-specific T cells, the effector function of these cells, T cell proliferation in general, and the recruitment of cells into the draining lymph nodes. This delay does not appear to be the result of general immunosuppressive effects of the infection, a limitation in parasite numbers, or parasite trafficking to lymph nodes or to the specific epitope. Increasing the initial infecting dose or the density of parasite epitopes on APCs can modestly speed the generation of anti-T. cruzi T cell responses. Given these characteristics of the response, we propose that T. cruzi is a stealth invader, largely avoiding recognition by components of the innate immune system until the infection is well established. This conclusion is supported by the ability to accelerate the induction of T cell responses to T. cruzi by administration of ligands for TLR2 and TLR9 at the time of infection. These studies highlight a previously unappreciated mechanism of immune evasion, the surreptitious establishment of infection, by the protozoan T. cruzi. The Journal of Immunology, 2009, 183: 1245–1252.

The protozoan Trypanosoma cruzi is the etiological agent of Chagas disease and affects millions of people mainly in the poorest regions of Latin America. The main transmission route in nature is thought to be through the deposition of the feces of the hematophagous insect vector onto the skin following a blood meal. Parasites then enter the hosts through injuries on the skin or the mucosa. It has been shown in experimental models that 15 min of direct contact of infected feces with the puncture site of a bite from the insect vector is sufficient to allow for infection (1).

CD8+ T cells have been shown to be crucial in the control of T. cruzi (2, 3), and in mice, this response can be monitored using MHC class I tetramers containing epitopes encoded by T. cruzi trans-sialidase proteins (4). The response against the immunodominant TSKB20 epitope in C57BL/6d mice occupies fully 30% of the total CD8+ T cell population at its peak, which is an incredibly robust level considering the complex genome of T. cruzi that encodes over 12,000 distinct proteins including thousands of trans-sialidase family proteins (5).

Despite the generation of this vigorous CD8+ T cell response, as well as other immune effector mechanisms, T. cruzi is controlled but rarely completely cleared, resulting in persistent infections. It is also noteworthy that although the immune response to T. cruzi is both potent and effective, it appears to be rather slow to develop, with the peak level of T. cruzi-specific CD8+ T cells occurring some 3 wk after the initial infection. This relative delay in immune response is distinct from that observed in other viral, bacterial, or protozoal infections, in which the highest level of specific CD8+ T cells is often obtained within 5–9 days of infection (6). In this study, we investigated the potential causes of this delay in the early generation of T cell responses to T. cruzi and conclude that the ability of the parasite to establish infection without an early triggering of innate immune recognition is the primary culprit.

Materials and Methods

Mice, parasites, and infections

C57BL/6J (B6) mice (CD45.2) were purchased from The Jackson Laboratory or bred and maintained in our animal facility under specific pathogen-free conditions. RAG knockout OT-I TCR (CD45.1) transgenic mice were obtained from Dr. K. Klonowski (University of Georgia, Athens, GA). Tissue culture trypomastigotes of the Brazil and Y strains of T. cruzi were obtained from passage through Vero cells. T. cruzi Y strain parasites were transfected with a pTREX plasmid (7) containing the coding sequence of the OVA gene fused to the N-terminal portion of the T. cruzi gpl72 gene to generate OVA-expressing parasites. For the in vivo proliferation assays, naïve RAG knockout OT-I (CD45.1) CFSE-labeled splenocytes were i.v. transferred into C57BL/6d mice (CD45.2) 24 h before infection with 105 T. cruzi-OVA trypomastigotes. Listeria monocytogenes expressing OVA (6) was obtained from Dr. K. Klonowski (University of Georgia, Athens, GA) and 106 bacteria per mouse were i.v. injected. All animal protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

Bone marrow-derived dendritic cell (DC)3 culture and infection

DC were derived from bone marrow cells cultured with GM-CSF (20 ng/ml) plus IL-4 (10 ng/ml) and matured with anti-CD40 (1 μg/ml) or LPS (10 ng/ml). DC were infected overnight with trypomastigotes (5:1 ratio of parasite to DC), pulsed for 1 h with TSKB20 peptide (ANYKFTLV, 10 μM), or left unstimulated. A total of 2 × 105–106 cells were transferred into the footpad or i.v. delivered in naïve B6 mice.

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3 Abbreviations used in this paper: DC, dendritic cell; PRR, pattern recognition receptor.

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T cell specificity and cytokine production

Spleen suspensions and draining lymph nodes were mechanically disrupted, and single cell suspensions were stained with TSKB20/KΩ tetramer as previously described (4) or were stimulated overnight with 1 μM of TSKB20 peptide and processed with a Cytotox/Cytoperm kit (BD Pharmingen) and stained to detect IFN-γ production in accordance with the manufacturer’s instructions and as previously described (8).

In vivo cytotoxicity assay

Spleen naive cells were incubated with 10 μM TSKB20 peptide or no peptide and labeled with 5 and 0.5 μM CFSE (Molecular Probes), respectively. Combined cells (2 × 10⁶) were i.v. transferred 18 h before killing and harvesting of spleens and draining lymph nodes. Specific killing was determined as described (4).

BrdU incorporation

Mice were i.v. injected with 1 mg of BrdU (Sigma-Aldrich) at 24 h before killing. Spleen and draining lymph node cells were processed with a BrdU detection kit (BD Pharmingen) following the manufacturer’s instructions.

Real-time PCR

Draining lymph node, site of infection, spleen, skeletal muscle, heart, and axillary lymph node samples from mice infected into the footpad with 1000 T. cruzi trypomastigotes were collected at 1, 4, and 10 days and analyzed by real-time PCR as previously described (9).

TLR agonists

TLR agonist for TLR2 (Pam3CSK4, 10 μg), TLR3 (polyinosinic-polycytidylic acid, 25 μg), TLR7 (gardiquimod, 2 μg), TLR9 (ODN 1826, 10 μg) from InvivoGen and TLR4 (50 μg LPS; Sigma-Aldrich) were dissolved following the manufacturer’s instructions and injected in the footpad along with 10⁷ T. cruzi trypomastigotes.

Cytokine/chemokine analysis

Serum samples from individual B6 mice infected with 10³ trypomastigotes in the footpad were kept at −20°C and tested in duplicate using the Luminox-based mouse cytokine/chemokine premixed LINCoPlex kit (Millipore). The cytokines and chemokines assayed were IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, TNF-α, IFN-γ, GM-CSF, MIP-1α, MCP-1, KC, RANTES, IP-10, and G-CSF.

Statistical analysis

Statistical significance was calculated using the two-tailed Mann-Whitney nonparametric test.

Results

T. cruzi-specific CD8⁺ T cells are slow to appear in draining lymph nodes

Lymph nodes draining sites of infection are important points of interaction between naive T cells and APCs and are the major locations at which CD8⁺ T cells are primed, including the case of infections vectored by insects (e.g., Plasmodium injected by the bites of mosquitoes (10)). In the natural infection by T. cruzi, parasites transmitted by the vector insects are not directly inoculated in the host, but rather are deposited on the skin within bug feces, and then manage to traverse the epithelial barrier through the mucosa or via breaks in the skin. Therefore it is logical that by this natural infection route, the lymph nodes draining the site of the infection would be the first place where the priming of parasite-specific T cells would be detected. To simulate the natural route, mice received a low-dose infection (1000 trypomastigotes) by s.c. injection in the footpad and the generated T cell response was examined in the draining lymph node and in the spleen. As shown in Fig. 1A, CD8⁺ T cells specific for the immunodominant TSKB20 peptide became detectable only after more than 9 days of infection and, as expected, were initially at higher frequency in the draining lymph node than in the spleen. Induction of effector function in the parasite-induced T cells followed similar kinetics, as indicated by TSKB20 peptide-induced IFN-γ production in CD8⁺ T cells and spontaneous IFN-γ production by non-CD8⁺ T cells (Fig. 1B) as well as the cytolytic activity for TSKB20-pulsed spleen cells (Fig. 1C).

As the kinetics of the observed TSKB20-specific response were substantially slower than that reported for multiple viral, bacterial, and protozoal infections (6, 11, 12), we considered the possibility that T cells of a more diverse specificity might dominate the early response to T. cruzi. To investigate this possibility, we next examined more general markers of activation in the total CD8⁺ population to identify the earliest time point at which any activation in this compartment could be detected. CD8⁺ T cell expression of CD69, a marker known to be up-regulated within a few hours after activation, exhibited a slight increase at 9 days postinfection in the draining lymph node and at later time points in the spleen (Fig. 1D). Likewise, the proliferation of CD8⁺ T cells as measured by either the incorporation of BrdU (Fig. 1E) or by the dilution of CFSE (Fig. 1F) was apparent no earlier than 9 days postinfection.

To investigate the possibility that a low precursor frequency of T. cruzi-specific T cells could account for the slow initial response to infection, we used the transfer of naive OVA-specific (OT-I) TCR transgenic CD8⁺ cells into naive mice to increase the precursor level of T cells capable of responding to T. cruzi transfectants expressing OVA (T. cruzi-OVA). We first demonstrated that infection with T. cruzi-OVA induces a robust SIINFEKL-specific T cell response in naive mice, with kinetics similar to that previously reported for the TSKB20-specific response (4) (Fig. 2A). Listeria-OVA infection of recipients of OT-I T cells resulted in substantial proliferation of the donor cells by 3 days and activation of nearly 100% of these cells by 7 days postinfection (Fig. 2B). However, no and only modest activation, respectively, was observed at 3 and 7 days in recipients infected with T. cruzi-OVA. Collectively these results demonstrate that although the CD8⁺ T cell response to T. cruzi infection is very robust, it is nevertheless relatively slow to develop in comparison to other well-studied infection models.

T. cruzi DNA is detected in the draining lymph node at 24 h postinfection

One possible explanation for the relatively slow generation of T cell responses following s.c. infection with T. cruzi is that the infective parasites remain at the local site and fail to traffic to the draining lymph node. To test this hypothesis we analyzed by real-time PCR the site of infection, the draining lymph node and the spleen at different time points to determine when parasites reach these secondary lymphoid organs. Parasite DNA was detected at the site of injection, and in the draining lymph node at 24 h after infection (Fig. 3) and in the spleen but not the skeletal muscle, heart, and axillary lymph nodes at 10 days postinfection (data not shown). The levels of parasite DNA increased over time at the site of infection and in the draining lymph node, suggesting active parasite proliferation or continued transport to these sites. To confirm the presence of live parasites in the lymph node at 24 h postinfection, collagenase-digested draining lymph nodes of mice infected in the footpad 24 h previously were transferred to highly susceptible IFN-γ knockout mice (13). One of three IFN-γ-deficient mice receiving cells from the draining lymph node became infected, whereas no infections were detected following transfer of the nondraining axillary lymph nodes from the same mice (data not shown). The detection of parasite DNA and parasites in the draining lymph nodes within 24 h of infection strongly suggests that the slow development of T. cruzi-specific T cell responses is not due to a failure of parasites to reach this potential site of T cell priming.
Parasite burden influences the timing of the CD8\(^+\) T cell response

The slow generation of CD8\(^+\) T cell responses after \(T. cruzi\) infection could also be due to a limiting parasite Ag load in our model of infection using \(10^3\) parasites. To determine the influence of parasite infective dose on the development of the anti-\(T. cruzi\) T cell response, we infected mice with higher (\(10^7\) parasites) and lower (50 parasites) doses and determined the time at which the TSKB20 tetramer-positive population became detectable. TSKB20 tetramer-binding cells were evident as early as 6 days postinfection in the mice infected with \(10^7\) parasites, nearly 5 days earlier than in mice infected with \(10^3\) parasites. Mice infected with 50 parasites did not have an evident tetramer-positive CD8\(^+\) population at 11 days postinfection (Fig. 4A).

The Brazil strain of \(T. cruzi\) used in our studies typically displays a parasitemia peak ~25 days postinfection, whereas the Y strain usually shows a peak of parasites in blood at 8 days postinfection (14) (Fig. 4B). A possible explanation for the delay in the CD8\(^+\) T cell response in our model was that Brazil strain has a relatively slow rate of proliferation and release from cells, thus delaying the release of parasite Ags available for priming. However, despite the earlier initial appearance (6 days) and significantly accelerated peak in parasites in the blood (8 days), the CD8\(^+\) population specific for TSKB20 was still not detected until 9 days postinfection with Y strain parasites (Fig. 4C). These results suggest that a higher infection level or infection with a strain with more rapid growth kinetics modestly hastens the development of the CD8\(^+\) response to \(T. cruzi\).

**FIGURE 1.** \(T. cruzi\)-specific responses are not detected until more than 9 days postinfection. B6 mice were infected in the footpad with \(10^5\) tryptomastigotes. CD8\(^+\) cells positive for TSKB20/Kb (A) and CD69 (D) were determined in spleen and draining lymph nodes \((n = 3\) mice). Data are mean ± SEM. \(*, p < 0.05\). Cells are gated in the CD4\(^+\)CD11b\(^-\)B220\(^-\) population and the percentage of CD8\(^+\)TSKB20/Kb\(^+\) cells of the total CD8\(^+\) population is shown. B, Spleen and lymph node cells were stimulated with TSKB20 peptide and stained for CD8\(^+\) and IFN-\(\gamma\). The percentage of CD8\(^+\) IFN-\(\gamma\)-producing cells in the total CD8\(^+\) population is shown. C, Naïve spleen cells pulsed with TSKB20 peptide (5 \(\mu\)M CFSE) and unpulsed (0.5 \(\mu\)M CFSE) were cotransferred into mice at different times postinfection and detected in spleen and draining lymph nodes 18 h later. The percentage of specific lysis is shown. E, BrdU was injected at different times after infection and 24 h later BrdU incorporation was determined in spleen and draining lymph node cells. Cells are gated in the CD4\(^+\)CD11b\(^-\)B220\(^-\) population, and the percentage of CD8\(^+\)BrdU\(^+\) cells in the total CD8\(^+\) population is shown. F, Naïve B6.SJL (CD45.1) CFSE-labeled splenocytes were i.v. transferred into B6 mice (CD45.2) 24 h before infection with \(10^5\) wild-type tryptomastigotes. Cells are gated in the CD8\(^+\)CD45.1\(^+\) population, and the percentage of cells from the original sample, which divided, is shown. Flow charts are representative of two to four separate experiments.

**FIGURE 2.** Response to \(T. cruzi\)-OVA infection is also delayed. A, \(T. cruzi\)-OVA parasites \((10^5)\) were inoculated i.p. in naïve B6 mice and the specific CD8\(^+\) response was determined by staining of peripheral blood with SIINFEKL-containing MHC class I tetramers. Data are ± SEM, for \(n = 3\) mice. B, RAG\(^{-/-}\) OT-I splenocytes were CFSE stained and transferred to naïve B6 mice 24 h before infection with \(10^5\) \(T. cruzi\)-OVA i.p. or \(10^5\) Listeria-OVA i.v. Cells are gated in the CD45.1\(^+\)CD8\(^+\) population.

Parasite burden influences the timing of the CD8\(^+\) T cell response

The slow generation of CD8\(^+\) T cell responses after \(T. cruzi\) infection could also be due to a limiting parasite Ag load in our model of infection using \(10^3\) parasites. To determine the influence of parasite infective dose on the development of the anti-\(T. cruzi\) T cell response, we infected mice with higher (\(10^7\) parasites) and lower (50 parasites) doses and determined the time at which the TSKB20 tetramer-positive population became detectable. TSKB20 tetramer-binding cells were evident as early as 6 days postinfection in the mice infected with \(10^7\) parasites, nearly 5 days earlier than in mice infected with \(10^3\) parasites, whereas mice infected with 50 parasites did not have an evident tetramer-positive CD8\(^+\) population at 11 days postinfection (Fig. 4A).

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Increased parasite/epitope abundance and access to APCs induces a more rapid TSKB20-specific CD8\(^+\) response

Although parasites are detected in the draining lymph node soon after \textit{T. cruzi} infection, they may nevertheless not efficiently induce T cell responses if their Ags are not adequately acquired and presented by professional APCs. To negate this possibility, bone marrow-derived, LPS-activated DC infected with trypomastigotes of \textit{T. cruzi} were transferred into the footpads of naive mice. Over 60% of these DC used in these experiments were infected, and this population was capable of inducing IFN-\(\gamma\) production by spleen cells obtained from \textit{T. cruzi}-immune mice, demonstrating their competence as APCs (data not shown). These \textit{T. cruzi}-infected DC induced detectable TSKB20-specific cells as early as 8 days postinfection, which is 3 days earlier than when equivalent numbers of parasites were injected directly into the footpad (Fig. 5A). Exposure of the infected bone marrow-derived DC to exogenous TSKB20 peptide before their injection into mice further accelerated the development of the TSKB20-specific T cell response, making it detectable at 6 days postinfection (Fig. 5B). Likewise, i.v. injection of \textit{T. cruzi}-infected DC also resulted in a TSKB20-specific response detectable at \(\sim\)6 days postinfection (see supplemental Fig. 1).\(^4\) These results suggest that multiple factors, including access to and the activation status of APCs, the epitope density on APCs, and the overall parasite burden, combine to influence the rate of generation of anti-\textit{T. cruzi} CD8\(^+\) T cell responses.

There is no a general immunosuppressive effect of \textit{T. cruzi} on CD8\(^+\) T cell priming

\textit{T. cruzi} is often described as being generally immunosuppressive (15–18). To investigate the possibility that \textit{T. cruzi} infection might generally interfere with the generation of T cell responses, we coinfections either mice or DC with wild-type \textit{T. cruzi} and \textit{Listeria}-OVA and monitored the timing of induction of CD8\(^+\) T cell response to OVA SIINFEKL and \textit{T. cruzi} TSKB20. OVA-expressing \textit{Listeria} induced strong CD8\(^+\) T cell responses by 6 days postinfection, irrespective of the coinfection with \textit{T. cruzi} (6) (Fig. 6). Likewise, the timing of the tetramer-specific responses

\(^4\) The online version of this article contains supplemental material.
to SIINFEKL or TSKB20 following injection of infected bone marrow-derived DC was not altered by coinfection (see supplemental Fig. S2). These results strongly suggest that Ag presentation and the generation of T cell responses in general are not adversely affected by coinfection with *T. cruzi*.

**Induction of innate immune responses by *T. cruzi***

In the absence of any apparent deficit in T cell precursor frequency and activation or in Ag presentation function in *T. cruzi*-infected cells, we considered whether the relative delay in generation of T cell responses might be linked to a failure of the initial infection to activate crucial innate immune responses. The earliest measurable response to the footpad infection with 1000 parasites was a statistically significant increase in cell numbers in the draining lymph node at 6 days postinfection (Fig. 7A). Because we had not detected proliferation in this site before day 9 (Fig. 1, E and F), these results suggested an active recruitment of cells to this site of early Ag presentation. To determine whether the inflammatory mediators that are likely responsible for this recruitment could be detected systemically, the sera of infected mice were assayed for the presence of 22 cytokines and chemokines. The serum levels of chemokines CXCL10 (IP-10), CCL5 (RANTES), TNF-α, CXCL1 (KC), and CCL2 (MCP-1) as well as IL-5 showed a steady increase (Fig. 7, B–G) with significant levels above background evident by 8 (CXCL10 and TNF-α), 11 (CXCL1 and CCL2), and 14 (CCL5 and IL-5) days postinfection. TNF-α levels also showed a transient increase at 2 days postinfection (Fig. 7D). IFN-γ levels were below the detection limit of the assay during the majority of the time points becoming just detectable (but not significantly increased) at 8 days postinfection (Fig. 7H). The concentration of the remaining cytokines and chemokines tested (MIP-1α, GM-CSF, IL-1β, IL-1α, G-CSF, IL-2, IL-6, IL-7, IL-10, IL-12, IL-9, IL-13, IL-15 and IL-17) was not altered or remained below the detection level during the first 2 wk after infection.

A failure of the initial infection by *T. cruzi* to substantially activate innate responses was further supported by the demonstration that selected TLR agonists coinjected with parasites significantly accelerated the expansion of *T. cruzi*–specific CD8+ T cells relative to parasites alone. Combinations of TLR agonists have been shown previously to synergistically activate APCs leading to a higher expression of costimulatory molecules and IL-12 (19, 20). Mice injected with the combinations of TLR9 plus TLR2, TLR9 plus TLR3, and TLR7 plus TLR3 agonists revealed a substantial CD8+ T cell response specific for the TSKB20 peptide as early as 9 days postinfection and TLR9+TLR3/CD8+ T cell precursors. Results are representative of three separate experiments.

**FIGURE 6.** *T. cruzi* and *L. monocytogenes* coinfection does not influence the timing of the CD8+ T cell responses. SIINFEKL/Kb+ and TSKB20/Kb+ CD8+ cells were determined in spleens of mice i.v. infected with *Listeria*-OVA (Lm), *T. cruzi* (tryp), or with both pathogens together. Results are representative of three separate experiments.

**FIGURE 7.** Induction of cytokines and chemokines in blood occurs after 6 days postinfection. The total cell number in spleen and draining lymph nodes (A) and systemic concentration of CXCL1 (B), CXCL10 (C), TNF-α (D), CCL5 (E), IL-5 (F), CCL2 (G), and IFN-γ (H) were determined in mice infected with 10^3 parasites in the footpad. Data are ± SEM for n = 5 mice. *, p < 0.05.

**FIGURE 8.** The CD8+ response against *T. cruzi* can be hastened by the coinjection of TLR agonists. TSKB20/Kb+ CD8+ cells were determined in draining lymph nodes after coinjection of combinations of TLR agonists and parasites in the footpad. Results are representative of three separate experiments.
perhaps as early as day 7 in the case of the TLR9 and TLR2 combination (Fig. 8). Other combinations of TLR agonists such as TLR2 plus TLR7 and TLR4 plus TLR7 (data not shown) had no effect. Thus, infection with T. cruzi appears to induce a moderate innate immune response, perhaps contributing to a delay in the generation of the parasite-specific T cell responses.

Discussion

T. cruzi infection induces a remarkably strong and focused CD8\(^{+}\) T cell response that is crucial for the control of this intracellular pathogen. However, despite its potency, the initial development of this protective CD8\(^{+}\) T cell response in T. cruzi-infected mice is strikingly slow. CD8\(^{+}\) T cell responses have been well-studied in a number of viral, bacterial, and protozoal infections. In the cases of some of the better studied models (e.g., LCMV, Listeria monocytogenes, and Plasmodium yoelii) substantial pathogen-specific CD8\(^{+}\) T cell responses are detected by 3–6 days postinfection and peak by 8–9 days (6, 11, 12, 21). In contrast, we had previously observed that the T cells comprising the immunodominant T. cruzi-specific CD8\(^{+}\) response in mice were rarely even detectable before day 10 of infection (4). Others had observed similar slow kinetics for T cell responses in a variety of mouse models of T. cruzi infection and alternative metrics (14, 22). Therefore, T. cruzi is among a select set of pathogens with the capacity to invade and proliferate but with a delayed induction of pathogen-specific T cell responses (23).

In this study we have examined in detail the generation of the T. cruzi-specific CD8\(^{+}\) T cell response and addressed possible explanations for this apparent delayed induction of protective T cell responses. Typically, adaptive immune responses against infections originating in the skin are mounted in the lymph nodes draining the site of pathogen entrance. Even for malaria, an infection characterized by a rapid colonization of the liver, it has recently been shown that the CD8\(^{+}\) T cell response originates in the lymph nodes draining the site of parasite entrance at the skin and not in the lymph nodes draining the liver as was previously thought (10). Using a system that attempts to simulate some of the characteristics of one natural route of infection (e.g., s.c. inoculation of low number of parasites), we failed to detect a T. cruzi-specific CD8\(^{+}\) response, as measured by TSKB20 tetramer staining, IFN-γ production and cytotoxic activity, before 10 days postinfection. Further investigation of other indicators of T cell activation beyond those specific for these immunodominant epitopes revealed that activation (as indicated by expression of CD69) and proliferation in the T cell compartment was also not evident until more than 9 days postinfection. Although this study focused primarily on CD8\(^{+}\) T cell responses, it is also clear that activation in the non-CD8\(^{+}\) compartment has similar kinetics.

A number of possible factors in the lag in the generation of CD8\(^{+}\) T cell responses, such as the route of infection, the virulence of different parasite strains, and the Ag/epitope specificity and precursor frequency of the T cells were all ruled out as major contributors. OVA-expressing T. cruzi also induce a SIINFEKL-specific T cell response that is even more potent than the already robust response to the endogenous TSKB20 peptide, but this response is equally slow in its initial induction and is only marginally accelerated by increasing the number of naive OVA-specific T cell precursors. Parasite strains with faster kinetics of expansion in vivo, as well as less virulent lines (data not shown) also failed to provoke a substantially more rapid T cell response.

A general immunosuppressive effect by T. cruzi on immune responses was also not responsible for the slow initial T cell response. Despite the evidence for highly effective control of T. cruzi infection in immunocompetent hosts, and the clear generation of potent parasite-specific T and B cell responses, responses to T. cruzi infection are often referred to as “suppressed” and “nonspecific” (15–18). However, not surprisingly, coinfections with Listeria and T. cruzi resulted in unaltered kinetics of T cell responses to both pathogens and DC coinfected with these pathogens induce similar responses as singly infected DC. A generalized immunosuppression is also incongruent with the eventual strength of the CD8\(^{+}\) T cell response in this infection (e.g., how could active regulation delay the response but fail to dampen what becomes one of the most potent CD8\(^{+}\) T cell responses reported in any infectious disease?).

The inherent growth rate of T. cruzi could be argued to be the primary reason for a slower induction of T cell responses in this infection—relative to the speed of viral and bacterial infections such as LCMV and Listeria. And indeed the ability to accelerate the development of the T cell response by delivering higher infectious doses of T. cruzi might support this argument. The potential for a more rapid spread of the bacterial and viral pathogens should also allow their Ags to more quickly reach sites of priming for the CD8\(^{+}\) T cell response. Both RT-PCR and lymph node tissue transfer experiments confirm that a low number of T. cruzi reach the lymph node draining sites of infection within 24 h, demonstrating that parasites are not absolutely sequestered at the infection site. Other investigators have reported the detection of parasite DNA in the draining lymph nodes of mice within 3 days after conjunctival infection with T. cruzi (24). Furthermore, low dose infection with the protozoan P. yoelii is reported to yield detectable CD8\(^{+}\) T cell responses within 1-2 days, rivaling the speed of numerous viral and bacterial systems (11). Thus the fact that protozoans like Plasmodium and T. cruzi are slower growing relative to viruses and bacteria cannot alone account for the sluggish development of the T cell response in this infection. Increasing the infective dose by 4 log values, to a physiologically unnatural level of 10\(^{5}\) trypanosomes in the footpad, accelerates the generation of the TSKB20-specific response, but still not approaching what is observed in Listeria infection (6, 12).

How is it that T. cruzi can avoid triggering the more rapid T cell response characteristic of many other infections? The timing of the appearance of the initial response relative to the infection cycle of T. cruzi may provide the first hint of an answer to this question. In vitro observations suggest that the period from T. cruzi infection of a host cell to the destruction of that cell and the release of several hundred parasite progeny is on the order of 4–5 days. The first indication of a T cell response following infection with a “rele vant” parasite load, to a model parasite Ag and in the presence of a high frequency of T cell precursors (i.e., infection with 1000 T. cruzi-OVA in mice receiving transferred OT-1 T cells), is ~7 days. In contrast, the response to Listeria under similar conditions is readily evident by 3 days, which is a difference of 4 days, the approximate time needed for T. cruzi to complete a first round of replication in mammalian hosts. Thus, we believe that the relatively slow initial kinetics of the T cell response to T. cruzi is best accounted for by concluding that the primary invasion is relatively “silent” and that cellular immune system recognition of the infection does not occur until this first round of replication is complete and the expanded population of parasites is released. The kinetics from this point are similar to that reported for multiple other infections; responding T cells are observed ~4 days later (day 9 of T. cruzi infection) and the peak response is reached within an additional 5–10 days.

Several pieces of data presented herein provide support for this model. First the pace of accumulation of cells in the lymph nodes draining the sites of infection as well as the detection of systemic
levels of inflammatory cytokines and chemokines provide the earliest indication of immune system detection of the infection; increases in both of these parameters are evident after more than 6 days postinfection, exactly as expected if these responses are elicited by the death of the infected host cells and the release of an expanded population of parasites following the first round of cell entry and parasite proliferation. CXCL10 and TNF-\(\alpha\) levels are significantly increased at 8 days postinfection, followed by CCL5 (RANTES), CXCL10 (IP-10), CXCL1 (KC), CCL2 (MCP-1), IFN-\(\gamma\), and IL-5. In contrast, in Listeria infection, an early production of CCL3 (MIP-1a), CCL2, and CCL5 is detected in serum at 3 days postinfection (25) and splenocytes producing these chemokines as well as IFN-\(\gamma\) can be detected without restimulation by 1 day postinfection (26). Similarly, IFN-\(\gamma\) and CCL5 concentrations in serum increase at 4–6 days postinfection with P. berghei and P. yoelii (27, 28).

The fact that the kinetics of the T cell response to T. cruzi can be accelerated by the codelivery of ligands for several TLRs also supports a model that T. cruzi initially fails to activate host pattern recognition receptors (PRR). Interestingly, the most effective of the sets of TLR ligands in terms of hastening the development of anti-T. cruzi T cell responses are those targeted for TLR2 and TLR9, the very TLRs with which T. cruzi GPI anchors and DNA, respectively, have been shown to interact (29, 30). It is perhaps not surprising that T. cruzi would fail to trigger these particular TLRs upon initial infection, as neither its GPI anchors nor its DNA would be expected to be exposed on viable, infecting trypanosomes. We propose that this compartmentalization of potential TLR agonists prevents the activation of host cells (including potential professional APCs) during the initial infection by T. cruzi and thus contributes to the relative delay in the generation of CD8\(^+\) responses.

If T. cruzi behaves in vivo as it does in vitro (31), then the initially unimpeached replication of T. cruzi would result in a several hundred-fold increase in the number of parasites released in an infected host within 4–5 days after infection. This increase in parasite load, the release of parasite byproducts from within the infected host cells, as well as the death of the host cell and the revelation of damage-associated molecular patterns (reviewed in Ref. 32) would all be expected to facilitate innate immune recognition of “danger” that is considered necessary for the initiation of adaptive immune responses. The modest acceleration in the kinetics of T cell responses by high-dose initial infection (Fig. 4A) could be explained by an increase in some (e.g., parasite numbers and possible host exposure to dead parasites or parasite products), but not all (host cell death) of these factors.

A model of “stealth infection” by T. cruzi is also supported by in vitro analysis of the response of host cells to infection by T. cruzi. Again, in contrast to a number of other pathogens, the response of human foreskin fibroblasts to T. cruzi, even at a 10:1 parasite to host cell ratio is remarkably low (33). A 10:1 parasite to host cell exposure of HEK cells resulted in TLR9-dependent activation, but even at a 50:1 parasite to host cell ratio, no colocalization or interaction of T. cruzi with TLR9 was evident. This result prompted the conclusion that the death or killing of T. cruzi by host cells was required to release agonists of TLR9 (34). Macrophages and DC appear to have a more potent PRR-dependent responses to T. cruzi (35), related perhaps to their higher expression and diversity of PRR and to their enhanced phagocytic and cytotoxic activity relative to other host cells. The ability of T. cruzi to invade many different host cells other than these phagocytic cells, as well as to quickly exit phagosomes, might also assist in avoiding early immune system recognition.

It could be argued that the delay we observe in generation of pathogen-specific T cell responses is too short to be of significant consequence for a chronic infection like T. cruzi. However, despite the fact that this infection is persistent, immunity to T. cruzi in most cases is actually incredibly effective, eventually controlling the infection to the point that directly demonstrating parasite persistence is quite difficult. This potent immune control, although clearing infection and preventing reinfection in most tissues, allows for the persistence of parasites in a few crucial sites (e.g., muscle, adipose tissue, nervous system). We propose that the stealth entry of T. cruzi into hosts, and the consequent delay in the generation of T cell responses is an important contributor to the initial establishment of the infection, and most importantly, in allowing the parasites to reach sites where it persists, such as muscle and fat, before a strong adaptive immune response is mounted. T. cruzi then appears to have some selective advantage for persistence in these tissues. Although the nature of this tissue-specific persistence is not known, it could have an immunologic (36) or metabolic (37) basis.

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Disclosures
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SUPP. FIG. S1. Delivery of infected DC speeds the development of the CD8⁺ T cell response. Bone marrow derived DC were infected overnight with trypomastigotes (5:1 parasites : DC ratio) and i.v. transferred into naïve B6 mice. TSKB20/Kb⁺CD8⁺ cells were determined in spleens at different times post infection. Results are representative of 2 separate experiments.

SUPP. FIG. S2. DC co-infection with T. cruzi and L. monocytogenes does not alter the timing of CD8⁺ T cells responses. Bone marrow derived DC were infected overnight with T. cruzi (5:1 parasite : DC ratio) and/or for 1 h with L. monocytogenes (30:1 bacteria : DC ratio) before i.v. transfer in naïve B6 mice. TSKB20/Kb⁺ CD8⁺ cells were determined in spleen samples of mice receiving DC infected with T. cruzi (tryp), L. monocytogenes (LM) or both pathogens together. Cells are gated in the CD4⁻CD11b⁻ B220⁻ population.