Immunoendocrinology of the Thymus in Chagas Disease

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Abstract
During immune response to infectious agents, the host develops an inflammatory response which could fail to eliminate the pathogen or may become dysregulated. In this case, the ongoing response acquires a new status and turns out to be detrimental. The same elements taking part in the establishment and regulation of the inflammatory response (cytokines, chemokines, regulatory T cells and counteracting compounds like glucocorticoids) may also mediate harmful effects. Thymic disturbances seen during Trypanosoma cruzi infection fit well with this conceptual framework. After infection, this organ suffers a severe atrophy due to apoptosis-induced thymocyte exhaustion, mainly affecting the immature double-positive (DP) CD4+CD8+ population. Thymus cellularity depletion, which occurs in the absence of main immunological mediators involved in anti-T. cruzi defense, seems to be linked to a systemic cytokine/hormonal imbalance, involving a dysregulated increase in TNF-α and corticosterone hormone levels. Additionally, we have found an anomalous exit of potentially autoimmune DP cells to the periphery, in parallel to a shrinkage in the compartment of natural regulatory T cells. In this context, our data clearly point to the view that the thymus is a target organ of T. cruzi infection. Preserved thymus may be essential for the development of an effective immune response against T. cruzi, but this organ is severely affected by a dysregulated circuit of proinflammatory cytokines and glucocorticoids. Also, the alterations observed in the DP population might have potential implications for the autoimmune component of human Chagas disease.

Introduction

Chagas disease is a tropical neglected disease caused by an intracellular protozoan parasite, Trypanosoma cruzi. In Latin America, around 12 million people are infected, with 20,000 annual deaths [1]. Currently, the disease has gained relevance in the US and Europe due to migration of infected people [2]. Infection with T. cruzi usually results in a silent acute phase. When clinically evident, the disease manifests with fever, lymphadenopathy, hepatosplenomegaly or major complications such as myocarditis or meningoencephalitis. Moreover, parasites are evident in the circulation and several host tissues in-
including myocardial and skeletal muscles, nervous system as well as lymphoid and endocrine organs. In humans, the spontaneous recovery from the acute phase occurs around 2 months after infection. Upon that, the patient enters into a long indeterminate latent stage without symptoms and scarce parasitism. The latent infection may remain silent for the rest of one’s life, although one third of fatally infected patients further develop clinical symptoms such as chronic cardiac dysfunction, colon or esophagus megadisease [3]. In individuals with chronic T. cruzi infection, mortality is mainly due to heart failure as a consequence of a protracted and destructive myocarditis named ‘chronic chagasic myocardopathy’, which seems to result from both autoimmune reactions and parasite persistence in the heart tissue [4, 5].

In a broad sense, the host’s response to infectious agents involves the generation of an inflammatory response addressed to eliminate the pathogen, promotes tissue repair and restores functionality and tissue homeostasis. When this response fails to eliminate the pathogen, the ongoing adaptive response acquires a new status and may become detrimental. In this way, the same elements taking part in the establishment and regulation of the inflammatory response [cytokines, chemokines, regulatory T cells and counteracting compounds like glucocorticoid hormones (GCs)] may also mediate harmful effects. Many disturbances seen during T. cruzi infection, in humans or experimental models, fit well with this conceptual framework.

Considering the usefulness of infecting murine hosts with T. cruzi, as a tool for studying the immunological mechanisms underlying disease development, we will focus herein on a series of thymic alterations accompanying the acute murine infection, including the severe thymic atrophy secondary to a massive death of double-positive (DP) CD4+CD8+ T cells and the escape of potentially autoreactive immature cells from the thymus to the periphery.

It is presently established that thymic output of lymphocytes continues throughout life [6, 7], for which output changes during the infection may influence disease outcome. As such, thymus alterations during chagasic infection should not be simplistically viewed as a bystander phenomenon, but as a relevant component in disease pathology. Indeed, an understanding of the mechanisms and mediators underlying thymocyte depletion will help in the further design of rational therapies addressed to reverse these changes and potentially improve immune function.

Normal T cell development depends on complex cell-cell interactions among subsets of developing thymocytes and microenvironmental cells [8–10]. Furthermore, the cytokines secreted by these cells provide the signals mediating some of the main events involved in the physiological control of thymocyte development. In the course of T. cruzi infection, there is a depletion of thymic lymphocytes that results from multiple interactions, in which products from the host’s response, including cytokines and chemokines, as well as an imbalanced immunooendocrine response play a role.

Among a large series of mediators playing a role in normal intrathymic T cell development as well as in disease-associated alterations, our discussion will focus mainly on TNF-α and GCs.

### The Thymus and Its Alterations during Acute Experimental Chagas Disease

The thymus is a target organ in many pathological situations, such as physical stress, infections and malignancies, and one common feature is a thymic atrophy [11, 12]. As stated above, a series of studies [13] showed that acutely T. cruzi-infected mice reveal an important degree of thymic atrophy (fig. 1a). Moreover, representative histological studies of C57BL/6 thymuses taken at different days following infection (see representative data in fig. 1b) showed a progressive depletion of cortical thymocytes, also evidenced by the loss of corticomedullary boundaries, reaching a severe degree of atrophy 3 weeks after infection [14, 15]. This contrasted with the enlargement of subcutaneous lymph nodes and spleen seen in the same animals, thus pointing to a compartmentalization of T cell alterations during the acute phase of the infection [16].

Mice controlling acute infection (BALB/c strain) showed a recovery of thymic histological architecture. Histology of the infected thymus revealed a loss of cortical thymocytes with loss of corticomedullary boundaries (fig. 1c). Moreover, we observed the presence of numerous apoptotic bodies with apoptosis being confirmed by gel electrophoresis of DNA and TUNEL staining [14]. Flow cytometry studies with annexin V plus propidium iodide also revealed an important apoptotic death of DP cells, more pronounced in susceptible than resistant strains of mice [15]. In parallel, other mechanisms of cell loss can be implicated, such as an increase in the release of cells from the organ or a diminution in proliferation and low income of bone marrow-derived precursors [17]. Taken together, these data clearly point to the notion that the microarchitecture and function of the thymus are affected during acute T. cruzi infection.
Role of TNF-α during T. cruzi Infection and Its Effect on the Thymus

TNF-α is a pleiotropic cytokine responsible for the induction of T lineage commitment and differentiation, as well as negative selection of immature DP population [18, 19]. Although the effects of TNF-α in the thymus are complex, support for a physiologic role of locally produced TNF-α in thymus development is the thymic hypertrophy seen in the TNF receptor (TNFR) knockout mice [20] and the abnormally small thymuses observed in mice overexpressing TNF-α [21].

While high TNF-α levels are involved in apoptosis of immature bystander thymocytes regardless of their antigen specificity [22], TNFR knockout mice exhibit aberrant negative selection [23]. Part of this apparently ambiguous behavior of TNF-α may result from changes in the expression of the two TNFR during thymocyte differentiation [24], as apoptosis is mediated by both TNFR-1 and TNFR-2, whereas proliferation is mediated solely by TNFR-2 [25, 26]. Additionally, TNF-α enhances the activity of regulatory T cells predominantly through TNFR-2, limiting a possible collateral tissue damage caused by excessive immune responses [27].
In addition, it is known that TNF-α not only plays a role in the control of acute T. cruzi infection in mice [28], but also exerts detrimental effects during the course of the disease [29]. Several clinical and experimental studies demonstrate an essential role of TNF-α in the host anti-T. cruzi defense, triggering phagocytic macrophage activation and inflammation [30, 31]. At the same time, elevated TNF-α levels are correlated with pathology, including excessive inflammation, wasting disease and death [32, 33]. In addition, our studies showed that in the course of acute T. cruzi infection, thymus atrophy is concomitant with systemic triggering of TNF-α [15].

The relevance of TNF-α in the acute T. cruzi infection has been supported by studies of cytokine neutralization or in TNF-α or TNFR knockout mice, in which a serious deficiency in host resistance to this protozoan, ameliorated cachexia and reduced myocardial inflammatory infiltrates were found [29, 34].

Proofs of the role of TNF-α were also obtained by recent studies in infected C57BL/6 and BALB/c mice with the Tulahuen strain of T. cruzi. Both strains developed an acute infection consisting in a marked parasitemia, myocardial inflammation and apoptosis-mediated thymocyte depletion. C57BL/6 mice showed a progressive fatal disease with a more profound thymocyte involution, whereas 60% of the BALB/c mice recovered. The severity of disease in the C57BL/6 mice was not linked to an increased parasite load, since parasitemia, myocardial parasite nests and amastigote counts in peritoneal macrophages did not differ from data recorded in BALB/c animals. By contrast, C57BL/6 mice exhibited an imbalance between pro- and anti-inflammatory cytokines with higher and lower blood levels of TNF-α and IL-10, respectively [15].

Because TNF-α levels in plasma are noticeably augmented during the acute phase, we considered the possibility that an apoptotic mechanism mediated by this cytokine was involved in deleting DP cells. Nevertheless, when we treated C57BL/6 mice undergoing T. cruzi infection with anti-TNF-α monoclonal antibody, the thymic atrophy was not modified. By contrast, protracted neutralization of this cytokine, essential to mount immune and inflammatory responses, resulted in increased parasitemia and myocardial amastigote nests, together with a reduced number and severity of the inflammatory heart lesions. Interestingly, similar results observed in transgenic mice expressing a fusion protein (TNFR1-FcIgG3) able to neutralize TNF-α effect in vivo also showed a higher parasite load and reduced myocardial inflammatory infiltrates upon T. cruzi infection [35]. In the same vein, thymuses from double-deficient mice for type 1 and type 2 TNF-α receptors exhibited a profound atrophy after infection with T. cruzi [16], confirming that this cytokine is not – at least directly – involved in thymocyte depletion. Strikingly, the compartmentalization of immune response against T. cruzi is evident in studies showing that in mesenteric lymph nodes TNF-α is involved in the apoptosis of the CD8+ T cell population [36]. Nevertheless, despite the ability of TNF-α to induce thymocyte apoptosis [32], the results discussed above indicate that modulators other than this cytokine are involved in the T.-cruzi-induced thymus atrophy. In this respect, it is noteworthy that, together with IL-1β and IL-6, at high concentrations TNF-α activates the hypothalamic-pituitary-adrenal (HPA) axis, initiating an endocrine response, which in turn influences the immune response [37].

**Involvement of GCs in Susceptibility versus Resistance in Chagas Disease**

It is well established that the immune, endocrine and central nervous systems are integrated through a network of signaling molecules (cytokines, hormones, and neurotransmitters), able to affect each other and hence constituting a critical integrated physiologic circuitry for the regulation and orchestration of a defensive response [38, 39]. As mentioned above, proinflammatory cytokines such as TNF-α, IL-1β and IL-6, released by activated immune cells, not only initiate immune reactions but also gain access through the circulation to the central nervous system, triggering a variety of neuroendocrine counter-regulatory mechanisms, i.e., the HPA axis activation. In fact, these cytokines stimulate the production of CRH in the hypothalamus which in turn leads to pituitary production of ACTH, followed by the secretion of GCs by the adrenal cortex. GCs exert both inhibitory and stimulatory effects on many facets of the immune response. For example, GCs inhibit the production of proinflammatory cytokines and favor a Th1/Th2 shift, serving to prevent a cellular mediated immune overshoot by counteracting the tissue-damaging effects of macrophages and Th1 cells [40–42]. In combating an infectious challenge, the host mounts an immune response, but when the magnitude of the immune response exceeds a certain threshold, harmful consequences ensue, with effects that antagonize or potentiate those of the immune response. During T. cruzi infection, there is a clear pathogenic correlation between immunoendoctrine abnormalities and disease outcome.
It is known that adrenal GCs have a strong influence on the thymus, as judged by the stress-related thymic involution, as well as by the rapid increase in thymocyte numbers after adrenalectomy [43] and the thymocyte apoptosis accompanying increased GC concentrations in blood [44].

GCs are also produced by thymic epithelial cells, pointing out the paracrine role of this hormone within the thymus [45]. Theoretically, GCs might be important not only for regulating the number and maturation of thymocytes, but also for influencing the functionality of the developing T cell repertoire, possibly by tuning the affinity level for antigen-MHC recognition [46].

Comparative studies in C57BL/6 and BALB/c mice revealed a differential corticosterone response following acute T. cruzi infection. Although both strains showed an intense stimulation of the HPA axis at 3 weeks of infection (a 42-fold increase with respect to the baseline levels in C57BL/6 mice), increase in circulating corticosterone levels occurred earlier in BALB/c mice, 2- to 3-fold higher at the end of the first week after infection [47]. Because endogenous GCs can inhibit the synthesis of proinflammatory cytokines, also inducing programmed cell death in thymocytes, C57BL/6 mice were further treated with RU486, a GC receptor antagonist [48]. Blockade of the GC receptor with RU486 resulted in a 50% reduction of thymic atrophy, shortened survival time in C57BL/6 mice, further enhancement of TNF-α levels in circulation and increased lethality to 100% in BALB/c mice [47]. Taken together, these data show that the HPA axis is stimulated during T. cruzi infection, and that interference with this endocrine response prevents thymus involution but aggravates the disease.

Extending these observations, IL-6 has also been associated with the enhanced activity of the HPA axis during T. cruzi infection (Colombian strain) in BALB/c mice. Infected mice showed increased systemic IL-6 levels, and supernatants of adenopituitary cell cultures challenged with the parasite also contained more IL-6. Interestingly, the enhanced production of corticosterone in infected animals was not associated with the concentration of ACTH, indicating some degree of dysregulation in the HPA axis [49].

Overall, these data point out that during T. cruzi infection, endogenous GCs released in response to TNF-α and other proinflammatory cytokines seem to be, at least partially, responsible for lymphoid tissue depletion in the thymic microenvironment. The main features of this immunoendocrine circuitry are described in figure 2.
Migration Patterns of Thymocytes and Peripheral T Cells during T. cruzi Infection

In addition to the thymic alterations occurring during *T. cruzi* infection, we have shown changes in the migration pattern of recently exported T cells, concomitantly with the presence of immature CD4+CD8+ cells in the periphery [17, 50]. These lymphocytes present increased levels of ECM receptors for fibronectin or laminin, which may favor thymocyte egress to the periphery [51]. In parallel, variations in the immunologic T cell repertoire, either by presenting forbidden T cell receptors [52], or changes in the proportions of this population were also found [Roggero E., unpubl. data], which might be an explanation for the autoimmune component seen in human and murine Chagas disease.

Several molecules may be related to changes in patterns of T cell migration. Some studies revealed that CXCL12-induced migration is increased during *T. cruzi* infection, either at the thymic level or at the sites of immune effector function [53]. Furthermore, TNF-α, which binds ECM ligands and synergizes with CXCL12, seems to be involved in the escape of immature cells from the thymus during acute infection [Pérez et al., in preparation]. In this context, it is important to bear in mind that TNF-α modulates the expression of molecules involved in lymphocyte recruitment during inflammatory reactions – such as chagasic myocarditis – in which cellular infiltrates comprise abundant numbers of T cells, many of them potentially autoreactive [54].

The role of TNF-α in heart tissue pathology is further exemplified by studies in *T.-cruzi*-infected mice disrupted from both TNFR (p55/p75 knockout): these animals are unable to generate an influx of inflammatory cells to the myocardium [16].

Strikingly, although preventing thymic atrophy, GC abrogation does not completely impede the escape of mature and immature thymic T cells to the periphery during infection, as ascertained by the number of FITC+ recent thymic emigrants detected in the spleen and lymph nodes (fig. 3).

Altogether, it seems clear that the complex intrathy mic interactions between cytokines and chemokines con jointly with ECM components alter the normal thymocyte migration patterns and the export of T cells from the thymus during *T. cruzi* infection.

Natural Regulatory T Cells and Thymus Atrophy in Acute *T. cruzi* Infection

There is a consensus that during parasitic diseases, parasite survival in host tissues seems to be related to the activity of the suppressor T cell population, especially regulatory T cells bearing the phenotype CD4+CD25+ FoxP3+. These cells seem to be important, both for the protection against autoimmune and chronic inflammatory reactions, suppressing the immune response, but allowing parasite persistence [55]. Nevertheless, the role of regulatory T cells in Chagas disease is still controversial.

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**Fig. 3.** Abnormal thymic exportation occurs in the absence of GCs. Bilateral adrenalectomy (ADX) was performed 7 days before infection. Control mice were sham-operated. Recent thymic emigrants were detected by intrathymic FITC inoculation 24 h before. Mice were killed and secondary lymph organs (spleen and subcutaneous lymph nodes) were harvested. The number of CD3+FITC+ cells (a) or the percentages of CD4+CD8+FITC+ cells in these organs (b) were determined before and 14 days after infection. Results are expressed as mean ± SEM from 3–5 mice per group. A representative experiment from 2 independent series is shown. *p < 0.05, comparing infected versus noninfected counterparts.
As mentioned above, preserved thymus during *T. cruzi* infection in the mammalian host may be essential for the development of an effective immune response against parasites. In parallel, thymocyte loss may also impact on the tissue damage-related autoimmune events occurring during the chronic infection, given that the thymus is important for the generation of ‘natural’ T regulatory cells. However, there are still no available studies on the potential impact of infection upon the regulatory T cell population that develops in the thymus. So far, studies focused on the peripheral regulatory T cell population, which comprises a pool of ‘natural’ and ‘induced’ regulatory T cells. Recently, it was found that during the early stage of Chagas infection in children, the number of circulating CD4+CD25+ T cells decreases, whereas in the indeterminate stage there is a significant increase in their frequency [56, 57]. Other studies show an expansion of peripheral regulatory T cells during acute experimental infection, which does not seem sufficient to contain the inflammatory response and the associated pathology [58, 59]. Interestingly, Mariano et al. [60] observed a decrease in survival of infected animals when they are depleted of peripheral regulatory T cells, suggesting a role of these cells in resistance to infection.

Recent studies showed that experimental *T. cruzi* infection also impacts thymic ‘natural’ regulatory T cells. Infection caused by different parasite strains (Tulahuen or Colombian) in either susceptible or resistant mice resulted in a clear depletion of natural regulatory T cells. Confirming previous data [61], in uninfected animals, thymic FoxP3+ cells are located within the medulla and the corticomedullary junction (fig. 4a), being absent in the thymic cortex, since FoxP3 is expressed in the CD4+ single-positive stage. In acutely infected mice, their location was maintained predominantly in the medullary region, although a few of them can be seen in the cortex (fig. 4b). Importantly, their absolute number is notably lower in infected than in control mice, and such loss seems to occur by apoptosis [Calmón-Hamaty F., Pérez A.R., in preparation].

Nevertheless, the link between the autoimmune component in Chagas disease and the loss of regulatory T cells during the acute phase, together with potential migratory alterations in FoxP3+ cells, remain to be investigated more profoundly.

**Fig. 4.** Localization of FoxP3+ cells in the thymus of control and *T. cruzi*-infected C57BL/6 mice. The figure corresponds to micrographs from confocal microscopy showing the distribution pattern of FoxP3+ cells in the thymus of normal C57BL/6 mice (a), or mice challenged with *T. cruzi* (Tulahuen strain) 14 days after infection (b). FoxP3+ cells (red; for colors, see online version) are located within the medulla (M), where epithelial cells are revealed by staining with anti-cytokeratin antibody (green). This distribution pattern of FoxP3+ cells is also seen in the thymus of infected C57BL/6 mice. However, it is clear that in infected thymus, there are few FoxP3+ cells in the cortex of the thymic lobule. The arrows indicate FoxP3+ cells and bars show the magnification of each micrograph.
Other Molecules Involved in *T. cruzi* Induce Thymic Atrophy

The flow of T lymphocytes to the different compartments of the immune system, and sites of immune effector activity is tightly regulated by cytokines and chemokines. Plenty of findings show that these molecules have an important role in regulating T-cell-mediated responses against parasite infection, and hence influencing host resistance. Early Th1 response in the periphery after *T. cruzi* infection is characterized by the production of IL-12 by macrophages, allowing NK cells to synthesize IFN-γ, which in turn acts synergistically with TNF-α inducing NO production, through inducible NO synthase (iNOS), to cope with parasites [63–65]. On the other hand, Th2 cytokines are necessary to counteract excessive inflammatory response, but when they are secreted at high levels, they may aggravate the course of infection [66]. While in *T. cruzi* infection, Th1- and Th2-type cytokines are associated with resistance and susceptibility, respectively [67], their influence upon the *T. cruzi*-induced thymic atrophy has not been properly analyzed.

To screen the potential role of different mediators or their receptors in infection-associated thymic alterations, we analyzed thymus cellularity, percentage of DP cells and apoptosis at the time when changes are clearly evident, namely 2 weeks after *T. cruzi* (Y strain) challenge. As illustrated in figure 5, we evaluated mice knocked out for the following key molecules: IL-12, IFN-γ and iNOS (proinflammatory pathways), IL-4 and IL-10 (anti-inflammatory cytokines) and CCL3 and CCR2 (chemotactic response). We found that thymocyte cellularity is similar between noninfected IFN-γ- and iNOS-deficient mice with a C57BL/6 background compared with the wild type ones. Nevertheless, a clear loss of DP thymocytes by apoptosis is observed in the infected deficient
mice, showing that thymic atrophy is independent of IFN-γ or iNOS (data not shown).

By contrast, thymocytes were more preserved in IL-12-deficient mice, suggesting that IL-12 is involved in the death of immature T cells (fig. 5a). Moreover, percentages of DP thymocyte subpopulations seem to be preserved 14 days after infection (infected animals: DP cells = 89%, CD4+ cells = 6%, CD8+ cells = 2%, double-negative cells = 3%). Evaluation of apoptosis corroborated these observations (annexin V+/propidium iodide+ thymocytes: infected IL-12 knockout mice = 8 ± 2.0%; infected wild-type mice = 18 ± 3.5%).

As regards the participation of the ‘anti-inflammatory’ cytokines (fig. 5b), we observed that noninfected IL-4-deficient mice showed a slightly higher thymus cellularity compared with the wild type, but exhibited a similar atrophy after infection. Strikingly, noninfected IL-10 knockout mice showed a significant reduction in the total number of thymocytes compared with the wild-type animals, suggesting that IL-10 is necessary for an adequate thymus development. Nevertheless, T. cruzi infection also led to a severe decrease in thymic cellularity.

Chemokines and chemokine receptors from the CC family have been reported to be involved in the normal thymus development [68] as well as the control of T. cruzi and chronic heart alterations [69–71]. At the thymic level, our results show that the basal number of thymocytes and thymic cellular loss after infection were similar in CCL3-deficient mice compared to wild-type mice (fig. 5c). In contrast, CCR2-deficient mice exhibited a great decrease in thymic cellularity compared with noninfected C57BL/6 mice, suggesting a critical role for some of its ligands (CCL2, CCL7, CCL8, CCL12, CCL13) in normal thymus physiology (fig. 5c). Nevertheless, these chemokines do not seem to be involved in thymocyte depletion caused by the infection.

Overall, these data show that thymic atrophy can be generated in the absence of key molecules involved in peripheral anti-T. cruzi resistance. At the same time, the partial protection of thymic atrophy observed in IL-12-deficient mice opens a new field of investigation.

Lastly, thymic atrophy induced by T. cruzi infection was also observed in Fas-deficient gld/gld and perforin knockout animals [36].

Conclusions

The results discussed herein suggest that the evolution of T. cruzi infection and its pathological consequences are not only due to immune-mediated mechanisms but also to a more generalized defensive response, also comprising endocrine axes, with important implications for the host-parasite relationship. Accordingly, the immune and endocrine exchange of information leading to a sort of regulatory response turns out to negatively influence the course of the disease. Actually, it favors the establishment of an adverse state characterized by important alterations in essential biological functions, including defective control of inflammation, catabolic-linked cachexia, and changes in several immune compartments which, in addition to their immediate repercussion on infection control, may also impact on the autoimmune phenomena seen as disease progresses (fig. 2).

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