Chagas disease is a Latin America zoonosis that is caused by the flagellate protozoan Trypanosoma cruzi (1). The most severe consequences of this disease occur in the chronic phase, with the establishment of digestive megasymphdrome and/or myocarditis, with death due to arrhythmia or congestive heart failure eventually occurring in the last case. Importantly, no spontaneous cure occurs in either humans or experimental models.

In spite of the development of a specific immune response that allows control of the acute infection, total elimination of T. cruzi does not occur (2), inasmuch as a small number of parasites remain, leading the infection to a state of chronicity. The incomplete clearance of T. cruzi is strictly related to the strategies developed by the parasite to evade the immune system, such as escaping from the parasitophorous vacuole to the cytosol in mononuclear phagocytes (3), evading complement-dependent lysis (4, 5), interfering with the intracellular signaling pathway of infected structural cells (6), and hijacking transforming growth factor β (TGF-β) (7), or is due to the host deficiencies with respect to constraining the infection, such as the negative regulation of effector leukocytes that results in CD4+ T cell senescence and the loss of the effector activity of tissue-infiltrating CD8+ T cells (8, 9) and the immunomodulation of an anti-T. cruzi CD8+ effector T cell response (10, 11).

In the previous decade, government efforts designed for housing amelioration and vector control have led to a sharp reduction in T. cruzi infections and almost complete elimination of new cases in countries of endemicity such as Uruguay, Chile, and Brazil (12). These policies have also been implemented in other American countries, and it is expected that the peridomiciliar form of the infection will be eradicated in the near future. Nevertheless, estimates indicate that 8 million people have already been infected and that 30 to 40% will suffer from the life-threatening complications of chronic infection. The options for chronic patients are limited because specific chemotherapy treatments do not result in the positive outcomes observed in patients in the acute phase of the disease (1, 13). Therefore, there is a great need to develop a therapeutic vaccine that can increase the ability of the immune system to eliminate T. cruzi infection.

On the basis of these considerations, we sought to enhance the specific immune response by challenging chronically infected mice with homologous parasites. Previously, we showed that mice that were chronically infected with the Y strain of T. cruzi presented a reduction in the subpatent parasitemia levels and enhanced anti-T. cruzi effector mechanisms when they were intra-
venously (i.v.) challenged with live homologous parasites (14). No effect of the parasite challenge on the heart could be analyzed in those experiments, since T. cruzi Y parasites rarely cause chronic cardiac inflammation. Therefore, to address the latter issue, we evaluated here the effects of homologous parasitic challenge on C3H/HePAS mice that were chronically infected with Sylvio X10/4 parasites, an experimental model that shows strong chronic cardiopathy with many characteristics that resemble the human disease (15). Two opposite nonexclusive effects could be anticipated after the challenge; on the one hand, part of the injected parasites could colonize the host cells and increase tissue damage; on the other hand, they could also boost the immune response and facilitate T. cruzi elimination. Nevertheless, neither a single dose of homologous parasites nor a sustained four-dose challenge was able to cause reproducible changes over the course of the chronic cardiac inflammation.

MATERIALS AND METHODS

Mice, parasites, and infection. Six- to 8-week-old C3H/HePAS male and female mice were bred under specific, pathogen-free conditions at the Isogenic Mouse Facility of the Biomedical Sciences Institute of the University of São Paulo, São Paulo, Brazil. The T. cruzi Sylvio X10/4 clone (16) was maintained by in vitro infection of LLCMK2 cells. Mice were infected with 10^5 trypomastigotes by the intraperitoneal (i.p.) route. All experiments were carried out in accordance with the ethical guidelines for experiments with mice provided by the Animal Experimentation Brazilian College (COBEA), and the protocols were approved by the Health Animal Committee (CEEA) of the University of São Paulo.

T. cruzi antigen preparation. Sylvio X10/4 T. cruzi parasites in LLCMK2 culture supernatant were washed in sterile phosphate-buffered saline (PBS) and subjected to 20 freeze-thaw cycles. The antigen concentration was adjusted to 1 × 10^5 parasites/ml, and the preparation was stored at −20°C.

Peripheral blood and spleen cell suspensions. Blood mononuclear cells, isolated by Percoll gradient separation of heparinized blood obtained by cardiac puncture, and spleen cells, obtained from homogenized spleens, were maintained in RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (50 μM), L-glutamine (2 mM), sodium pyruvate (1 mM), and 3% heat-inactivated fetal calf serum (FCS). All supplements were purchased from Life Technologies. Cell number was estimated using a Neubauer chamber.

Cell phenotyping. A total of 1 × 10^6 spleen blood and mononuclear cells from individual mice were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, and Cy-chrome-labeled monoclonal antibodies (MAB) (BD Pharmingen) to CD4 (H129.19), CD8 (53-6.7), and B220 (RA3-6B2), respectively. Cells were analyzed by flow cytometry using a FACSCalibur flow cytometer and CELLQUEST software (Becton, Dickinson).

Intracellular staining of Fosp3. Spleen cells (1 × 10^6) were surface stained with fluorochrome-labeled anti-CD4 and anti-CD25 (PC61) MABs. The cells were permeabilized and intracellularly stained for FoxP3 according to the manufacturer guidelines (catalog no. 12-5773-82; eBioscience), washed, and analyzed by flow cytometry.

Cytokine analysis in culture supernatants. IFN-γ, tumor necrosis factor alpha (TNF-α), interleukin-2 (IL-2), IL-4, IL-5, and IL-10 levels in spleen cell culture supernatant after stimulation with a T. cruzi antigen dose equivalent to 2.5 × 10^5 parasites for 48 to 72 h were estimated by the use of a cytometric bead array (CBA) (BD Pharmingen) following manufacturer guidelines. Briefly, 50-μl aliquots of supernatant were added to a pool of capture beads specific for each cytokine. Subsequently, phycoerythrin-fluorochrome-labeled detection reagent was added to these mixtures. After a 2 h of incubation at room temperature in the dark, bead mixtures were washed, and the pellets were analyzed by flow cytometry. The cytokine concentration in the supernatant was determined by the mean fluorescence intensity in fluorescence detector 2 in relation to titration curves obtained for each cytokine.

Analysis of subpatent parasitemia. T. cruzi parasites in the blood of chronically infected mice were detected by triplicate cultures of 5-μl blood aliquots in axenic liver infusion tryptose (LIT) medium (1 ml/well) incubated at 28°C. Cultures were screened for epimastigote growth twice a week during 1 month.

Histopathology. Mice were deeply anesthetized and bled to death by cardiac puncture. The heart was collected, fixed in 10% formalin, and preserved in paraffin. Six 5-μm-thick nonconsecutive slides were stained with hematoxylin-eosin for microscopy. According to the intensity of infiltrates in different areas of the heart, the following scores were established: absent (0), discrete (1), moderate (2), intense (3), very intense (4), and severe (5).

Statistical analysis. Statistical analysis was performed with a t test and 2-way analysis of variance (ANOVA), using Graph Pad Prism 4.0 software. Differences between two groups were statistically considered significant when the P value was < 0.05.

RESULTS

Single homologous challenge boosts the immune response but does not affect cardiac lesion. Sylvio X10/4 is a low-virulence T. cruzi clone that yields no patent parasitemia in immunocompe-
Enhanced levels of both IgG2a and IgG1 anti-
T. cruzi antibody in chronic mice that were infected with Sylvio X10/4 previously reported (20), IgG2a was the dominant subclass of se-
CD3/CD28 MAbs (see Fig. S1C in the supplemental material). As
CD8 served 10 days p.c., but not by day 60 p.c. (Fig. 1A). Likewise, a
spontaneously or after anti-CD3/CD28 stimulus were also ob-
seen in the blood of challenged mice (see Fig. S1B in the supplemental material). Significant augmentations of the num-
ers of CD4+ and CD8+ splenocytes that were producing IFN-γ spontaneuously or after anti-CD3/CD28 stimulus were also ob-
served 10 days p.c., but not by day 60 p.c. (Fig. 1A). Likewise, a
transient increase in the frequency of CD4+ and CD8+ cells was observed in the blood, but, differently from the spleen, it was detected only after in vitro stimulation with anti-
CD3/CD28 MAbs (see Fig. S1C in the supplemental material). As
previously reported (20), IgG2a was the dominant subclass of se-
rum antibody in chronic mice that were infected with Sylvio X10/4 T. cruzi even after the parasite challenge (Fig. 1B). However, en-
hanced levels of both IgG2a and IgG1 anti-T. cruzi serum antibod-
ies were observed on day 10 p.c.; this effect was no longer observed by day 60 p.c. The kinetics of the anti-T. cruzi IgG1 and IgG2a antibody serum levels are shown in Fig. S1D in the supplemental material, where the challenge resulted in a brief alteration of anti-
body levels between days 7 and 30 p.c. In the experiment shown, the challenge-induced increase of IgG1 was more robust than that
observed with IgG2a.

Chronic mice that were challenged with a single dose of homolo-
gous parasites were then analyzed for the presence of cardiac
lesions. It is known that, during chronic infection with Sylvio
X10/4 parasites, C3H/HePAS mice develop an intense infiltration of mononuclear cells into the heart tissue that affects the pericar-
dium, endocardium, and myocardium accompanied by the occa-
sional presence of parasite nests (15). Challenging C3H/HePAS mice with homologous parasites did not modify the intensity of mononuclear cell infiltration in the heart after 10 or 60 days (Fig. 1C), which remained very high (nearly 4 points) according to our
score.

Effect of sustained challenge with homologous parasites on the immune response of chronically infected mice. We consid-
ered the possibility that continuous stimulation of the immune
system was necessary to modulate the inflammatory response in the cardiac tissue because challenging chronically infected mice with a single parasite dose resulted in a brief boost in the immune
response that did not affect the level of cardiac lesion (Fig. 1C). To
test this possibility, Sylvio X10/4 chronically infected C3H/HePAS
mice (infected for 3 to 4 months) were challenged with four doses
of 5 × 10⁶ parasites given at 3-week intervals. The first two doses
were given i.v., and the other two were given i.p. The effects of
these challenges were analyzed 1 to 2 months after the last dose.
Sustained challenge increased the number of CD4+ cells but not
the number of CD8+ cells in the spleen of chronically infected
mice (Fig. 2A). In addition, while sustained challenge did not af-
flect the spontaneous production of IFN-γ, it increased the num-
ber of IFN-γ-secreting CD4+ cells after in vitro stimulation with anti-
CD3/anti-CD28 MAbs (Fig. 2B). No major changes were ob-
erved after sustained challenge in the frequencies of CD4+, CD8+, and B220+ cells in the bloodstream. However, challenged
mice showed an increase in the frequency of CD8+ blood cells but not of CD4+ blood cells that produced IFN-γ after in vitro anti-
CD3/CD28 stimulation (see Fig. S2 in the supplemental material).

The spleen cell response in chronically infected mice subjected
to sustained antigenic challenge was further evaluated by measur-
Analyzed the specific IgG1 and IgG2a serum levels in unchallenged To indirectly evaluate the impact of a sustained parasite challenge parasite-specific CD4 memory cells in the blood. This reinforces the notion that challenge induces a boost of the specific immune response to infection.

**Sustained challenge promotes an increased production of both TH1 and TH2 cytokines, IgG1 antibody levels, and regulatory T (T\(_{REG}\)) cells.** Control of T. cruzi parasite infection requires a dominant TH1 response, characterized by the production of IFN-\(\gamma\) and a shift to IFN-\(\gamma\)-dependent IgG2a antibodies (21, 22). To indirectly evaluate the impact of a sustained parasite challenge on the effector cytokine pattern in chronically infected mice, we analyzed the specific IgG1 and IgG2a serum levels in unchallenged and challenged mice. Figure 3A shows that in challenged mice, the serum levels of anti-T. cruzi IgG1 antibodies were notably increased, while T. cruzi IgG2a antibody levels were increased in just one of the four experiments that were performed (data not shown).

Because the switch to the IgG1 isotype is promoted by IL-4, a cytokine induced at low levels during T. cruzi infection, we wondered if a sustained challenge would alter the cytokine profile of chronically infected mice. Spleen cells from challenged and unchallenged mice were stimulated for 48 to 72 h with T. cruzi antigen, and the levels of cytokines were measured by the CBA. We observed that the sustained challenge promoted an increase in the production of IFN-\(\gamma\) and IL-2 by the splenocytes (Fig. 3B). However, IL-2 levels were lower in unchallenged or challenged mice than in the naïve ones (Fig. 3B, horizontal bars). A similar level of TNF-\(\alpha\) production was observed in splenocyte cultures from challenged mice (Fig. 3B). Interestingly, sustained challenge promoted a significant increase in the production of the TH2 cytokines IL-10, IL-4, and IL-5. These results indicate that a sustained challenge boosts the immune response in a mixed TH1 and TH2 profile.

We also investigated whether regulatory cell subsets were expanded in challenged mice because both pro- and anti-inflammatory cytokines were induced by a sustained immunologic challenge. Spleen CD4\(^+\) cells from challenged and unchallenged mice were analyzed for the expression of Foxp3, a transcription factor that is characteristic of regulatory T (T\(_{REG}\)) cells. Figure 3C shows that sustained challenge promoted an expansion of T\(_{REG}\) cells, most of which displayed the IL-2 receptor alpha chain CD25 (data not shown). These results reinforce the notion that repeated stimulation with homologous parasites boosts the regulatory circuits.

**Effects of sustained challenge on subpatent parasitemia and cardiopathy in chronically infected mice.** Our next step was to

FIG 2 Analysis of CD4\(^+\) and CD8\(^+\) splenocytes in chronically infected mice that were subjected to sustained challenge with homologous T. cruzi parasite cells. Unchallenged mice, chronically infected mice, and chronically infected mice that were submitted to sustained challenge with homologous parasites were euthanized 1 to 2 months after the last challenge dose and the spleen cell suspensions analyzed for (A) total numbers of CD4\(^+\), CD8\(^+\), and B (B220\(^-\)) cells, (B) numbers of CD4\(^+\) and CD8\(^+\) cells producing IFN-\(\gamma\), either spontaneously or after in vitro stimulation for 12 h with anti-CD3/anti-CD28 MAb, and (C) frequencies of proliferating (CFSE\(_{LOW}\)) CD4\(^+\) and CD8\(^+\) cells in response to stimulation with anti-CD3 (α-CD3)/anti-CD28 MAb, T. cruzi antigen (Tc Ag), or medium alone (—). Data represent the results determined with unchallenged (●) and challenged (●) chronically infected mice. *, P < 0.05; **, P < 0.01; #, P < 0.001 (compared to unchallenged chronically infected mice). Representative experiments are shown.

FIG 3 Analysis of functional polarization in chronically infected mice that were subjected to sustained challenge with homologous T. cruzi parasite cells. Unchallenged mice, chronically infected mice, and chronically infected mice that were submitted to sustained challenge with homologous parasites were bled and euthanized 1 to 2 months after the last challenge dose and analyzed for (A) T. cruzi-specific serum IgG2a and IgG1 antibody levels (OD values, 1/12,000 dilution), (B) in vitro T. cruzi antigen-induced production of TH1 and TH2 cytokines by spleen cells, and (C) the numbers of CD4\(^+\) Foxp3\(^+\) cells in the spleen. Data represent the results determined with unchallenged (●) and challenged (●) chronically infected mice. In panel B, the horizontal bars represent the mean values of cytokine levels in T. cruzi antigen-stimulated spleen cell cultures from noninfected mice. *, P < 0.05; **, P < 0.01; #, P < 0.001 (compared to unchallenged chronically infected mice). Representative experiments are shown.

**FIG 2** Analysis of CD4\(^+\) and CD8\(^+\) splenocytes in chronically infected mice that were subjected to sustained challenge with homologous T. cruzi parasite cells. Unchallenged mice, chronically infected mice, and chronically infected mice that were submitted to sustained challenge with homologous parasites were euthanized 1 to 2 months after the last challenge dose and the spleen cell suspensions analyzed for (A) total numbers of CD4\(^+\), CD8\(^+\), and B (B220\(^-\)) cells, (B) numbers of CD4\(^+\) and CD8\(^+\) cells producing IFN-\(\gamma\), either spontaneously or after in vitro stimulation for 12 h with anti-CD3/anti-CD28 MAb, and (C) frequencies of proliferating (CFSE\(_{LOW}\)) CD4\(^+\) and CD8\(^+\) cells in response to stimulation with anti-CD3 (α-CD3)/anti-CD28 MAb, T. cruzi antigen (Tc Ag), or medium alone (—). Data represent the results determined with unchallenged (●) and challenged (●) chronically infected mice. *, P < 0.05; **, P < 0.01; #, P < 0.001 (compared to unchallenged chronically infected mice). Representative experiments are shown.

**FIG 3** Analysis of functional polarization in chronically infected mice that were subjected to sustained challenge with homologous T. cruzi parasite cells. Unchallenged mice, chronically infected mice, and chronically infected mice that were submitted to sustained challenge with homologous parasites were bled and euthanized 1 to 2 months after the last challenge dose and analyzed for (A) T. cruzi-specific serum IgG2a and IgG1 antibody levels (OD values, 1/12,000 dilution), (B) in vitro T. cruzi antigen-induced production of TH1 and TH2 cytokines by spleen cells, and (C) the numbers of CD4\(^+\) Foxp3\(^+\) cells in the spleen. Data represent the results determined with unchallenged (●) and challenged (●) chronically infected mice. In panel B, the horizontal bars represent the mean values of cytokine levels in T. cruzi antigen-stimulated spleen cell cultures from noninfected mice. *, P < 0.05; **, P < 0.01; #, P < 0.001 (compared to unchallenged chronically infected mice). Representative experiments are shown.
evaluate the effect of sustained challenge with homologous parasites on subpatent parasitemia and cardiopathy of Sylvio X10/4 chronically infected mice. Four independent experiments were performed with 34 challenged and 30 unchallenged mice (Fig. 4). Subpatent parasitemias were estimated by determining the percentage of positive cultures in LIT medium using 5-μl blood aliquots from each mouse. In two of the experiments, chronically infected mice that were subjected to sustained challenge displayed a trend toward a decrease in subpatent parasitemia. In the other two experiments, the opposite result was observed, that is, an increase in parasitemia levels or a tendency for increased parasitemia after the four rounds of homologous challenge (Fig. 4A).

Similarly, the effects of a sustained challenge with homologous parasites on cardiac lesion were not consistent, since we observed a small decrease in global cardiopathy in two experiments, an augmentation of pericarditis and a tendency for higher global cardiopathy in a third experiment, and no major changes in the fourth experiment (Fig. 4B). Overall, our results indicate that sustained immunologic challenge does not consistently influence the inflammatory reaction in the heart of chronically infected mice. However, in spite of the variation observed in the results, a correlation was seen in the effects of the sustained challenge regarding the parasitemia and the cardiopathy. Thus, in the two experiments where the challenge decreased the cardiac compromise, a trend for lower parasitemia was observed. Additionally, in the experiment where the challenge enhanced the parasitemia, an increased level of pericarditis and a tendency for augmented global cardiopathy were evidenced.

DISCUSSION

The persistence of *T. cruzi* in the heart is considered the main underlying factor behind chronic chagasic cardiomyopathy (23–25). In consequence, a great number of experimental studies pursued the goal of reducing the parasite load as a means of diminishing or eliminating the chronic cardiopathy (26) by applying different approaches, such as prophylactic or therapeutic vaccines based on recombinant *T. cruzi* proteins (27, 28), naked parasite DNA (29), or recombinant viral vectors carrying *T. cruzi* genes (30–32).

To investigate the requirements to diminish the cardiac lesion in chronically infected hosts, we evaluated the effect of homologous challenge on C3H/HePAS mice that were chronically infected with Sylvio X10/4 parasites, a model that yields strong chronic myocarditis (14). We hypothesized that if parasite challenge reduced the systemic and the cardiac tissue parasite load in chronically infected mice, it should result in the long term in decreased cardiac inflammation and damage. We first evaluated the effects of challenge with a single parasite dose and observed increased specific serum IgG levels as well as the spontaneous IFN-γ production by CD4⁺ and CD8⁺ cells in the spleen and blood.
Nonetheless, these were transient boosts and were not noticeable 60 days after challenge. More importantly, we did not observe changes in the cardiopathy, with the intensity of mononuclear cell infiltrates seen in the cardiac tissue similar to that in unchallenged mice.

Bearing in mind that the immune-mediated destruction of parasites in the heart occurs preferentially following nest rupture and that the rupture of a significant number of nests is a process that takes a long period of time (33), we thought that the failure of single-dose challenge to modify the heart inflammation could be explained by the brevity of the boosted response. With the aim of inducing a long-lasting modification of the immune response, chronically infected mice were submitted to a sustained challenge with four parasite doses, and the effects on immune parameters were analyzed 1 to 2 months after the last dose. Sustained challenge promoted a significant increase in IFN-γ-producing memory CD4+ cells in the spleen and IFN-γ-producing memory CD8+ cells in the blood. It also increased the parasite-specific IgG.

Meanwhile, its effects on cardiac pathology were not consistent. Moreover, the effects of the sustained challenge in the levels of systemic parasitism differed in the different experiments. Yet, a correlation was observed in the effect of sustained challenge upon blood parasitism and cardiac pathology, a result that suggests a connection between the two parameters.

Different reasons can be hypothesized to explain why sustained challenge did not modify the cardiopathy, among which we should not discard the remote possibility of increased heart tissue infection by the newly inoculated parasites. More likely, we should consider that the boosted immune response in challenged mice was quantitatively and/or qualitatively insufficient to significantly diminish the levels of parasites that were present in the bloodstream or in the tissues. A qualitative defect is suggested by the fact that the boosted immune response of challenged mice shifted away from the protective TH1 response in chronically infected mice. Thus, the increase in T. cruzi-specific serum antibody levels occurred mainly for IgG1, an IL-4-dependent isotype (34) that shows lower opsonizing and complement activation capacities compared to IgG2a. Moreover, the boosted response included increases in both TH1 (IFN-γ, IL-2) and TH2 (IL-4, IL-5) cytokines, anti-inflammatory molecules (IL-10), and T REG cell population. A shift away from the TH1 lineage suggests that the host did not identify the challenge inocula as a dangerous stimulus that could easily be eliminated (35, 36). Destruction of the challenge inocula might have occurred because the injected trypomastigotes bound to specific IgG and were subsequently killed by phagocytic cells in the liver, lung, spleen, or peritoneal cavity, and the signal generated in this process might have not been sufficient to fully activate the immune response. Supporting this interpretation, we have previously shown that, following i.v. challenge of Y strain-infected chronic mice with homologous parasites, the liver parenchyma is infiltrated by a high number of memory CD8+ cells, but very few CD8+ effector cells (19). Another reason that could contribute to the absence of a consistent modification of the cardiopathy after sustained challenge is that the boosted immune response would be restricted to the blood, lungs, liver, and lymphoid organs (19); therefore, it impact upon the local inflammatory response in the heart could have been negligible. Thus, even if the levels of T. cruzi-specific IgG in the heart tissue would be similar to those observed in the serum, the same might not have occurred regarding the inflammatory cells (effector T cells, monocytes, etc.), since an increased local signaling would be required for an increased cell recruitment.

Due to the absence of the effect of a single-dose or a sustained immunologic challenge upon heart infiltrates, we may hypothesize that boosting the parasite-specific immune response of chronically infected mice with T. cruzi extracts or purified or recombinant antigens would not be relevant in the treatment of the chronic cardiopathy because, as in the case of the homologous challenge, the boosted response would not find the requirements for reducing the parasite load in the tissues. We suggest that, for a therapeutic vaccine to be effective in reducing the effects of the chronic disease, it might require the continuous provision of danger signals, a means of inducing a selective, long-lasting boost in IFN-γ-producing and cytotoxic CD8+ cells, a requirement that could be met by immunization with viral vectors containing T. cruzi parasite sequences (32, 37, 38). In this respect, it is important to mention the recent observation of Vasconcelos et al. (38) that a vaccination with an adenovirus vector carrying the ASP-2 T. cruzi gene was able to overcome the qualitative deficits in the specific CD8+ response of T. cruzi-infected mice.

Currently, various therapeutic vaccine candidates have proven that a reduction in the systemic and local T. cruzi parasite load and a reduction in heart tissue inflammation are possible (31, 39, 40). Hence, these studies must be pursued, as they represent, alone or in combination with specific chemotherapeutic, the most promising candidates for treating the complications of the chronic human disease.

ACKNOWLEDGMENTS

This study was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

We extend our thanks to Rogério Silva do Nascimento, Meire Ioshie Hiyane, and Bernardo Paulo Albe for technical assistance.

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