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Identification of Two Distinct Subpopulations of *Leishmania major*-Specific T Helper 2 Cells

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It is widely accepted that a strong Th2 response is responsible for nonhealing *Leishmania major* infections in BALB/c mice. This Th2 response has been thoroughly documented by measuring the levels of Th2 cytokines produced by CD4$^+$ T cells present in the lymphoid organs by enzyme-linked immunosorbent assay and PCR. However, the cytokine profile of *L. major*-specific Th2 cells has never been determined. In this study, we used the recently described Th2 marker T1/ST2 to characterize Th2 cells during the course of nonhealing *L. major* infection. We analyzed the intracellular cytokine profile of CD4$^+$ T1/ST2$^+$ T cells and showed that they clearly displayed a Th2 phenotype, as they expressed interleukin 4 (IL-4), IL-10, and IL-5. In addition, we detected another population of Th2 cells among the CD4$^+$ T1/ST2$^-$ T cells that expressed IL-4 and IL-10 but excluded IL-5. In summary, we show here that two type 2 subpopulations are present in the lymphoid organs of *L. major*-infected BALB/c mice; Th2 cells from both subsets expressed IL-4 and IL-10, but they could be distinguished by their expression of IL-5 and T1/ST2.

The selection of immune effector functions is controlled by antigen-specific T helper (Th) cells (17, 19, 27). Naive CD4$^+$ Th cells can differentiate from a common precursor cell into functionally distinct effector cells (15, 16) which mediate different immunological functions in protection and pathology in many infectious and autoimmune diseases. Based on their cytokine profiles, at least three subsets can be distinguished: Th0, Th1, and Th2. Th0 cells secrete interleukin-2 (IL-2), IL-4, and gamma interferon (IFN-γ), Th1 cells secrete IFN-γ, IL-2, and tumor necrosis factor beta, and Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13. In addition to the distinct cytokine profiles, transcription factors GATA-3 and T-bet are selectively expressed in Th2 and Th1 effector cells, respectively (30, 35, 36). The differentiation of naive CD4$^+$ T cells into Th1 or Th2 effector cells is a multistep process which is influenced by the environment in which the T cells react with antigen, e.g., the type of antigen-presenting cell, the concentration of antigen, the strength of T-cell receptor ligation, costimulation, major histocompatibility complex (MHC) and non-MHC genes, and the cytokine milieu (20, 27). However, the molecular mechanisms which drive this differentiation process are not fully understood.

Recently several groups reported that T1/ST2, an orphan receptor with homology to IL-1R, is a stable and selective marker of Th2 cells (9–11, 34), and the T1/ST2 molecule is also expressed on mast cells (14) and fibroblasts (4). T1/ST2 has been shown to be important for Th2 effector functions in several experimental models, as treatment with antibodies against the T1/ST2 molecule or with a T1/ST2 immunoglobulin fusion protein (T1-Fc) significantly reduced Th2-mediated effector functions (2, 11, 32). However, there are conflicting reports on the role of T1/ST2 in vivo since studies with T1/ST2 gene knockout mice showed that expression of the T1/ST2 molecule is not required for the differentiation and function of Th2 cells and mast cells (5, 28). Another study, also using T1/ST2 knockout mice, confirmed that T1/ST2 was not necessary for the in vitro differentiation of naive CD4$^+$ T cells into Th2 cells; however, it defined a key role for T1/ST2 in the early events involved in the generation of Th2 responses in vivo (31): the onset of primary lung granuloma formation around *Schistosoma mansoni* eggs was inhibited, and the levels of Th2 cytokines in immunized T1/ST2 knockout mice were reduced (31).

A recently reported role for T1/ST2 in human disease is based on results for patients suffering from bronchial asthma: the levels of soluble T1/ST2 in serum were significantly elevated during severe asthma attacks (22). In addition, high levels of T1/ST2 mRNA and protein have been correlated with low relapse risk in lymph node-negative breast cancer patients (25, 33).

Infection of mice with *Leishmania major* is widely used as a model to study the differential development and function of CD4$^+$ T-helper-cell subsets in vivo. The ability of different inbred strains of mice to heal *L. major* infection is correlated with induction of polarized Th1 responses, and nonhealing, progressive disease is correlated with polarized Th2 responses (3, 26). Although T1/ST2$^+$ cells have been implicated in nonhealing *L. major* infections (9, 34), the exact role of Th2 cells and the function of the T1/ST2 molecule are not understood.

The aim of this study was to characterize CD4$^+$ Th2 cells by using the T1/ST2 marker. The results presented here show

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TABLE 1. Primers for RT-PCR

Protein detected	Primer sequence	Fragment size (bp)
GATA-3	5′ ACGTCTCACTCTCGAGGCAGCATG 3′ GAAGTCCAGTCATGCAC	539
T-bet	5′ GAGCGGACCAACAGCATC 3′ CATCCACAAACATCCTGTAAT	483
HPRT	5′ GTAATGATCAGTCAACGGGGGAC 3′ CCAGCAAGCTTGCAACCTTAACCA	208

that, after infection of nonhealing BALB/c mice with *L. major*, naive T helper cells differentiate in vivo into at least two subpopulations of Th2 effector cells. These Th2 effector cells can be distinguished by the expression of T1/ST2 and of IL-5.

MATERIALS AND METHODS

Mice. BALB/c mice were purchased from Charles River, and 6- to 12-week-old females were used in all experiments. The animal colonies were screened regularly for mouse pathogens and consistently tested negative. Animal experiments were performed in accordance with institutional and Home Office guidelines.

Infections and monitoring of lesions. *L. major* LV39 (MRHO/SU/59/P strain) was isolated from skin lesions of infected mice and maintained as described previously (7). Infections with 2×10^6 stationary-phase *L. major* LV39 promastigotes were performed by subcutaneous injection into one footpad in a final volume of 50 μl. Lesion development was monitored weekly by measuring the increase in footpad thickness with a dial caliper (Kröplin Schnelltaster, Schlüchtern, Germany).

Lymphocyte cultures. (i) Primary stimulation. Spleen and lymph node cells (5×10^6/ml) were stimulated in the presence of 4×10^6 live *L. major* promastigotes/ml (rendered replication incompetent by UV irradiation) at 37°C in 5% CO_2 in air in 24-well Costar plates in a final volume of 1 ml of complete medium (Dulbecco's modified Eagle medium [Gibco] supplemented with 5% heat-inactivated fetal bovine serum [FBS; Gibco], 216 μg of L-glutamine/ml, 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES, 100 U of penicillin ml^{-1}, 100 μg of streptomycin/ml). In the experiments in which lymphoid cells were cultured under Th2 conditions, anti-IFN-γ (XMG1.2 [1]; 3 μg/ml) and recombinant mouse IL-4 (X63Ag8-653 [6]; 200 U/ml) were added to the cultures; in the experiments in which cells were cultured under Th1 conditions, anti-IL-4 (11B11 [21]; 10 μg/ml) and recombinant mouse IL-12 (Genetics Institute; lot no. MRB02894-1; 10 U/ml) were added to the cultures. Cells were cultured for the period of time indicated in the figure legends, and then the culture supernatants were collected and frozen prior to cytokine determination.

(ii) Secondary stimulation. Spleen cells (5×10^6/ml) were stimulated in the presence of 4×10^6 live *L. major* promastigotes/ml as described above. Six days later, the cells were harvested, a Ficoll gradient was performed, and $CD4^+$ T cells were purified by magnetic bead separation with anti-CD4 microbeads (Miltenyi Biotec), according to the protocol provided by the manufacturer. The purity of the $CD4^+$ T cells was determined by flow cytometry and was >98%. $CD4^+$ T cells were restimulated in the presence of *L. major*-infected antigen-presenting cells prepared as described previously (8) and incubated for 4 days before the culture supernatants were collected for cytokine determination.

Flow-cytometric analysis. Lymphoid cells were stimulated as described above. Six days later, the cells were harvested, a Ficoll gradient was performed, and $CD4^+$ T cells were purified by magnetic bead separation with anti-CD4 microbeads. The detection of intracellular cytokine was performed as described previously (24). Briefly, 1.5×10^6 cells were stimulated with 50 ng of phorbol 12-myristate 13-acetate (PMA; Sigma) and 500 ng of ionomycin (Calbiochem) or, as a control, in the presence of complete medium alone for 4 h, with 10 μg of brefeldin A (Sigma) for the last 2 h. Before surface labeling with anti-CD4 (clone H129.19 or RM4-5 [Pharmingen, San Diego, Calif.]) or anti-T1/ST2 (clone DJ8 [13 or Morwell Diagnostics]), cells were preincubated with 1 μg of rat anti-mouse monoclonal antibody CD32/CD16 (FcγII/III receptor; Pharmingen) to reduce nonspecific binding. Cells were washed, fixed with 2% formaldehyde (Sigma), and permeabilized with 0.5% saponin (Sigma) before the anticytokine antibodies or the isotype controls were added (anti-IL-4, clone BVD4-1D11; anti-IFN-γ, clone XMG1.2; anti-IL-10, clone JES5-16E3; anti-IL-5, clone TRFK5; appropriately labeled rat immunoglobulin G1 [Pharmingen]). The detection of the intracellular cytokines was done with an EPICS XL instrument (Beckman Coulter), and data were analyzed with Beckman Coulter Expo32 software.

Analysis of expression of GATA-3 and T-bet. (i) Isolation of $CD4^+$ T1/$ST2^+$ T cells and $CD4^+$ T1/$ST2^-$ T cells. Spleen cells (5×10^6 ml^{-1}) from BALB/c mice infected for 1 week were stimulated with 4×10^6 live *L. major* promastigotes ml^{-1}. Six days later, the cells were harvested, a Ficoll gradient was performed, and $CD4^+$ T cells were purified by magnetic bead separation with anti-CD4 microbeads (Miltenyi Biotec). $CD4^+$ T cells were labeled with anti-T1/ST2, and $CD4^+$ T1/$ST2^+$ T cells (purity, >99%) and $CD4^+$ T1/$ST2^-$ T cells (purity, 99.5%) were sorted with an EPICS ELITE ESP cell sorter (Beckman Coulter).

(ii) Reverse transcriptase PCR. Total cellular RNA was extracted from $CD4^+$ T1/$ST2^+$ T cells and $CD4^+$ T1/$ST2^-$ T cells with an RNeasy MiniKit (Qiagen) according to the protocol provided by the manufacturer. Reverse transcription was performed on total RNA (1 μg) by using Moloney murine leukemia virus (Life Technologies) with an oligo(dT) primer (Pharmacia).

Aliquots of cDNA derived from $CD4^+$ T1/$ST2^+$ T cells and $CD4^+$ T1/$ST2^-$ T cells were amplified for the detection of GATA-3, T-bet, and hypoxanthine phosphoribosyltransferase (HPRT) (Table 1).

The amplification program consisted in an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min, and then 72°C for 10 min.

The PCR products for GATA-3, T-bet, and HPRT were visualized by electrophoresis of an ethidium bromide-stained 1% agarose gel. Two controls were run concurrently with the experimental samples: a negative control containing all PCR reagents minus the template and a RNA template lacking the reverse transcription step.

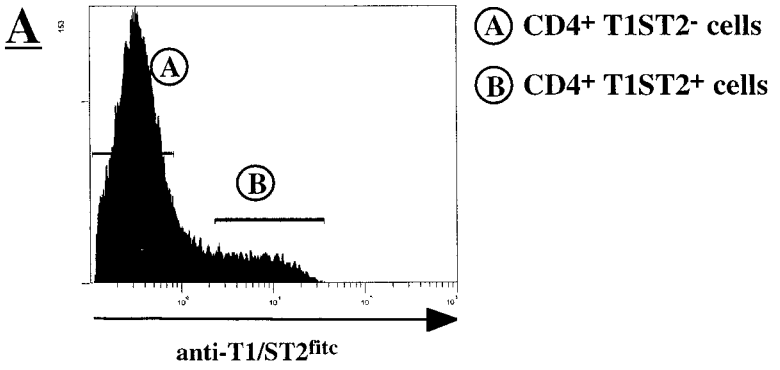
RESULTS

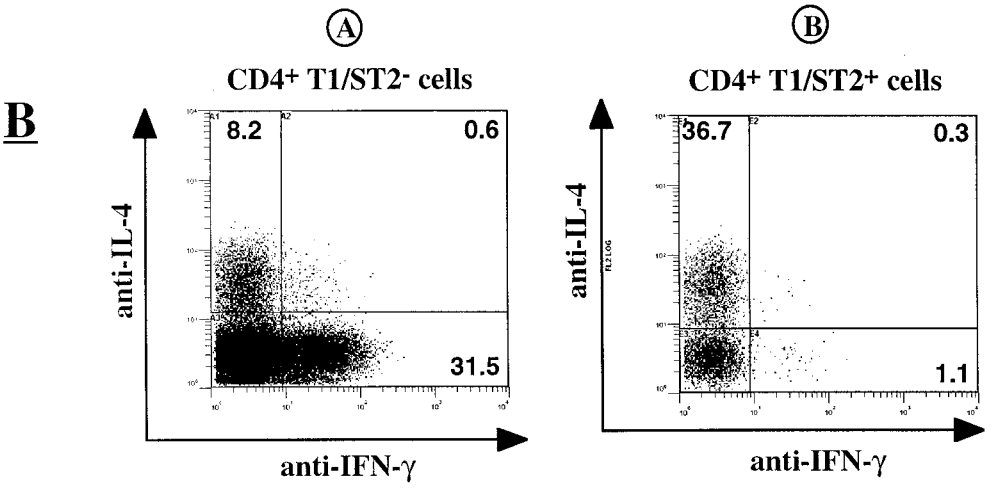
Cytokine profile of $CD4^+$ T1/$ST2^-$ T cells and $CD4^+$ T1/$ST2^+$ T cells isolated from *L. major*-infected BALB/c mice 1 week postinfection. To characterize *L. major*-specific $CD4^+$ T1/$ST2^+$ T cells isolated from BALB/c mice, we determined their cytokine profile by intracellular cytokine staining. We first established the kinetics of cytokine detection by $CD4^+$ T cells (IL-4, IL-5, IL-10, and IFN-γ) 1, 2, 4, and 6 days after antigenic restimulation in vitro. As presented in Table 2, IL-4, IL-5, IL-10, and IFN-γ were clearly detectable 6 days after in vitro antigenic restimulation. To assess the cytokine profile of Th2 cells, two regions were set up in *L. major*-specific $CD4^+$ T cells; the $CD4^+$ T1/$ST2^-$ population was gated in A (74.9%), and the $CD4^+$ T1/$ST2^+$ population was gated in B (14.5%) (Fig. 1A). The expression of T1/ST2 was not altered by PMA, ionomycin, and brefeldin A, as the frequencies of $CD4^+$ T cells expressing T1/ST2 before and after the polyclonal restimulation were similar (data not shown). Specificity controls are presented in Table 3. As shown in Fig. 1B, there are almost no $CD4^+$ T1/$ST2^+$ T cells expressing IFN-γ, compared to the

TABLE 2. Kinetics of cytokine detection in $CD4^+$ T cells[a]

Day post-restimulation	Frequency (%) of cytokine-expressing T cells			
	IL-4^+ $CD4^+$	IL-5^+ $CD4^+$	IL-10^+ $CD4^+$	IFN-γ $CD4^+$
1	0.5	0.2	0.1	2.7
2	0.8	0.3	0.3	2.5
4	2.6	0.7	0.5	9.6
6	4.3	3.1	2.1	29.7

[a] BALB/c mice were infected with *L. major* parasites, and, 1 week postinfection, the spleens were harvested and restimulated with antigen. At the indicated time points, cells were harvested, a Ficoll gradient was performed, and the cells were restimulated with PMA and ionomycin for 4 h. Brefeldin A was added to the cultures during the last 2 h. Cells were labeled with CD4 Cy-chrome, fixed, and permeabilized, the labeled anticytokine MAbs were added, and the frequencies of cytokine-expressing $CD4^+$ T cells were determined by flow cytometry.





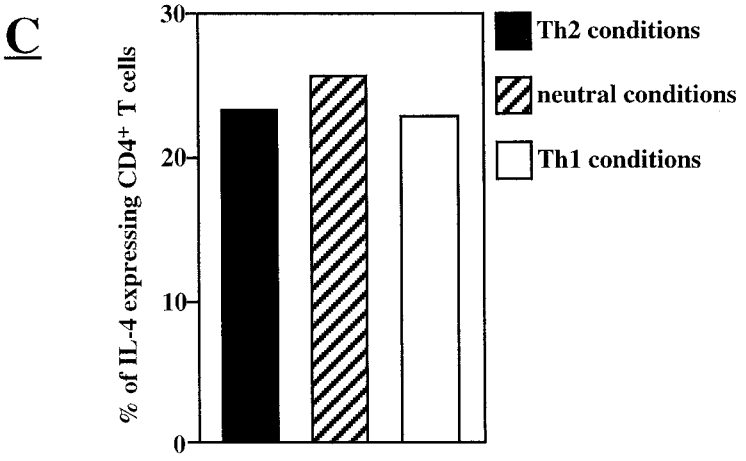


FIG. 1. Expression of IL-4 and IFN-γ in *L. major*-specific $CD4^+$ T cells. BALB/c mice were infected with *L. major* parasites and, 1 week postinfection, the spleens were harvested and restimulated with antigen. After 6 days, a Ficoll gradient was performed, $CD4^+$ T cells were purified by MACS, and restimulated with PMA and ionomycin in the presence of brefeldin A as described in Materials and Methods and labeled with anti-T1/ST2, anti-IL-4, and anti-IFN-γ. Gate A was set up on the $T1/ST2^-$ T cells, and gate B was set up on the $T1/ST2^+$ T cells (A). The frequencies of cytokine-expressing cells were determined by flow cytometry (B). (C) BALB/c mice were infected with *L. major* parasites and, 1 week postinfection, the spleens were harvested and restimulated in the presence of *L. major* alone (neutral conditions), *L. major* and IL-4 and anti-IFN-γ (Th2 condition), or *L. major* and IL-12 and anti-IL-4 (Th1 condition). After 6 days, the cells were harvested, a Ficoll gradient was performed, and $CD4^+$ T cells were purified by magnetic cell sorting (MACS) and were restimulated with *L. major*-infected antigen-presenting cells. Four days later, the cells were harvested, a Ficoll gradient was performed, and the $CD4^+$ T cells were restimulated with PMA and ionomycin in the presence of brefeldin A as described in Materials and Methods and labeled with anti-IL-4. The frequencies of cytokine-expressing $CD4^+$ T cells were determined by flow cytometry. The results of one out of three similar experiments are presented here.

TABLE 3. Specificity controls[a]

Isotype control or MAB	Frequency (%) of T cells with profile:	
	$CD4^+$ $T1/ST2^-$	$CD4^+$ $T1/ST2^+$
Isotype controls		
Anti-IgGl^{fitc}	0.2	0.8
Anti-IgGl^{pe}	0.9	1.1
MAbs		
Anti-IL-4^{pe}	1.4	1.0
Anti-IFN-γ^{fitc}	0.8	0.2
Anti-IL-10^{fitc}	0.3	0.5
Anti-IL-5^{pe}	0.3	0.6

[a] Spleen cells from BALB/c mice infected for 1 week were restimulated for 6 days in the presence of *L. major* parasites. After 6 days, $CD4^+$ T cells were isolated by MACS and stimulated in the presence (Isotype controls) or absence (MAbs) of PMA and ionomycin for 4 h. Brefeldin was added in both conditions during the last 2 h. Cells were labeled with anti-T1/ST2^{cc}, fixed, and permeabilized according to the protocol described in Materials and Methods. Appropriately labeled isotype controls (fluorescein isothiocyanate [^{fitc}] or R-phycoerythrin [^{pe}]) or anticytokine MAbs were added to the permeabilized cells, and the staining was analyzed by flow cytometry. Lymphoid cells from naive BALB/c mice were used as a control at the same time points, and no cytokines were detectable (data not shown). The expression of T1/ST2 was not altered by PMA, ionomycin, and brefeldin A, as the frequencies of $CD4^+$ T cells expressing T1/ST2 before and after the polyclonal restimulation (data not shown) were similar.

$CD4^+$ $T1/ST2^-$ T cells (1.4 versus 32.1%, respectively) but a high percentage of the $CD4^+$ $T1/ST2^+$ T cells express IL-4 (37%). These results indicate that *L. major*-specific $CD4^+$ T cells expressing T1/ST2 are Th2 cells, as they produce IL-4 but not IFN-γ. Surprisingly, another type 2 population was detected among the $CD4^+$ $T1/ST2^-$ T cells: 8.2% of the $CD4^+$ $T1/ST2^-$ T cells produce IL-4 but not IFN-γ. The expression of IL-4 was shown to be stable as the addition of IL-4 and anti-IFN-γ (Th2 condition) or IL-12 and anti-IL-4 (Th1 condition) to the culture only marginally altered the frequency of cytokine-expressing $CD4^+$ T cells during secondary restimulation (Fig. 1C).

To characterize *L. major* specific $CD4^+$ $T1/ST2^+$ T cells and $CD4^+$ $T1/ST2^-$ T cells further, the expression of other Th2 cytokines was determined. The presence of two distinct subpopulations of Th2 cells among the $CD4^+$ T cells was confirmed by the expression and coexpression of IL-10 and IL-4 by both $CD4^+$ $T1/ST2^+$ T cells and $CD4^+$ $T1/ST2^-$ T cells (Fig. 2A). The similar mean fluorescence intensities of cytokine expression (Fig. 2B) suggest that the amounts of IL-4 and IL-10 produced by cells from the two subpopulations of Th2 cells are similar. The coexpression of IL-4 and IL-5 in both Th2 populations was assessed, and the results are presented in Fig. 3. $CD4^+$ $T1/ST2^+$ T cells expressed each of these type 2 cytokines individually as well as simultaneously. In contrast, IL-5 could not be detected in the $CD4^+$ $T1/ST2^-$ T-cell subpopulation.

The results presented in Fig. 1 to 3 clearly show that $CD4^+$ T cells expressing T1/ST2 are Th2 cells. These data also indicate that, after *L. major* infection, two different populations of Th2 cells are generated in vivo and that they can be distinguished by their expression of the T1/ST2 molecule and IL-5.

Cytokine profile of $CD4^+$ $T1/ST2^-$ T cells and $CD4^+$ $T1/ST2^+$ T cells isolated from *L. major*-infected BALB/c mice 4 weeks postinfection. To determine whether the early cytokine pattern is stable and maintained during the course of cutaneous leishmaniasis, the intracellular cytokine profile of antigen-specific cells was assessed 4 weeks postinfection, a time when the nonhealing Th2 phenotype is completely established, and compared to that obtained 1 week postinfection. As presented in Fig. 4A, populations of IL-4-expressing $CD4^+$ T cells were detected in both the $CD4^+$ $T1/ST2^+$ T cells and $CD4^+$ $T1/ST2^-$ T cells derived from cultures from the popliteal lymph node cells (32.5 and 22.0%, respectively). Surprisingly, a small percentage of $CD4^+$ $T1/ST2^+$ T cells also express IFN-γ (4.8%) or IFN-γ and IL-4 (2.7%). An even higher percentage of Th0-like cells, i.e., $CD4^+$ T cells coexpressing IL-4 and IFN-γ, was detected among the $CD4^+$ $T1/ST2^-$ T-cell population (12.4%).

$CD4^+$ $T1/ST2^+$ T cells also express IL-10 alone (7.2%) or in combination with IL-4 (14.7%) (Fig. 4B). Type 2 cells were also present among the $CD4^+$ $T1/ST2^-$ T cells as shown by the expression of IL-10 (5.3%) or IL-4 and IL-10 (6.8%). Although at a lower frequency, a similar levels of expression or coexpression of IFN-γ, IL-4, or IL-10 were obtained with $CD4^+$ $T1/ST2^+$ T cells and $CD4^+$ $T1/ST2^-$ T cells isolated from the spleen cell cultures (data not shown).

In contrast to the results obtained with the $CD4^+$ $T1/ST2^+$ T cells from mice infected for 1 week (Fig. 3), intracellular expression of IL-5 was not detectable 4 weeks postinfection (data not shown) and it was low in both the $CD4^+$ $T1/ST2^+$ T cells (1.6%) and the $CD4^+$ $T1/ST2^-$ T cells from the lymph node cell cultures (1.1%; Fig. 4C).

In summary, the results presented in Fig. 4 show that two subpopulations of Th2 cells are present in established, nonhealing *L. major* infections.

Expression of GATA-3 and T-bet in $CD4^+$ $T1/ST2^+$ and $CD4^+$ $T1/ST2^-$ T cells. To verify the Th2 nature of the two subpopulations of Th2 cells, the expression of the Th2-specific transcription factor GATA-3 in the $CD4^+$ $T1/ST2^+$ and the $CD4^+$ $T1/ST2^-$ T cells was determined. In addition, the expression of T-bet, a Th1-specific transcription factor, was also determined. As shown in Fig. 5, GATA-3 is clearly expressed in the $CD4^+$ $T1/ST2^+$ T cells (Fig. 5A, lane 2) but T-bet could not be detected (Fig. 5B, lane 2), confirming the Th2 nature of this subset. GATA-3 was also detected in the $CD4^+$ $T1/ST2^-$ T cells (Fig. 5A, lane 1), further supporting the presence of a distinct population of $CD4^+$ $T1/ST2^-$ type 2 cells in *L. major*-infected BALB/c mice.

DISCUSSION

The results presented in this study show that *L. major*-specific $CD4^+$ $T1/ST2^+$ T cells isolated from BALB/c mice infected for 1 week displayed a clear Th2 phenotype: they coexpressed IL-4 and IL-10, IL-4 and IL-5, or IL-10 and IL-5 and did not express IFN-γ (Fig. 2 and 4). These results are in agreement with the study from Löhning et al. (10) showing that $CD4^+$ $T1/ST2^+$ T cells from the lungs of *S. mansoni*-infected mice unequivocally display a Th2 phenotype. The Th2 nature of *L. major*-specific $CD4^+$ $T1/ST2^+$ T cells was confirmed further by the results showing that they did not express T-bet (Fig. 5), a transcription factor selectively associated with Th1 cells (30), but expressed GATA-3 (Fig. 5), a transcription fac-

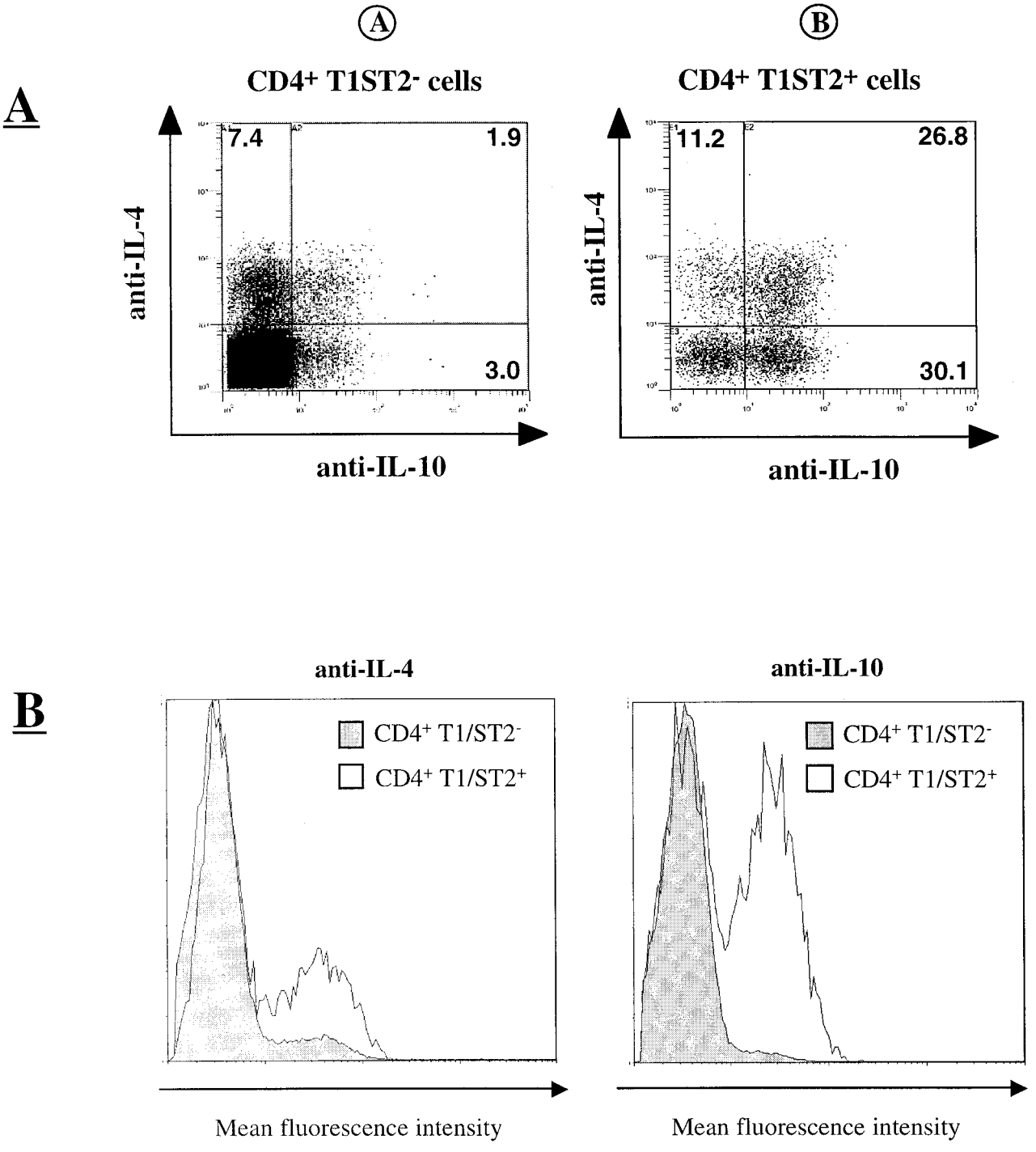


FIG. 2. Expression and coexpression of IL-4 and IL-10 in $CD4^+$ $T1/ST2^+$ and $CD4^+$ $T1/ST2^-$ T cells. (A) BALB/c mice were infected with *L. major* parasites and, 1 week postinfection, the spleens were harvested and restimulated with antigen. After 6 days, a Ficoll gradient was performed and $CD4^+$ T cells were purified by MACS and restimulated with PMA and ionomycin in the presence of brefeldin A as described in Materials and Methods and labeled with anti-T1/ST2, anti-IL-4, and anti-IL-10. Gate A was set up on $T1/ST2^-$ T cells, and gate B was set up on $T1/ST2^+$ T cells. The frequencies of cytokine-expressing cells were determined by flow cytometry. (B) White histograms, intensity of expression of IL-4 and IL-10 in $CD4^+$ $T1/ST2^+$ T cells; grey histograms, intensity of expression of IL-4 and IL-10 in $CD4^+$ $T1/ST2^-$ T cells. Both histograms are scaled to 100% of the peak values. The results of one out of three similar experiments are presented.

tor selectively expressed by Th2 cells (23). Although the relation between T1/ST2 expression and GATA-3 is not well established, it is possible that GATA-3 directly regulates the transcription of T1/ST2; indeed, naive $CD4^+$ T cells from CD2–GATA-3 transgenic mice displayed an aberrantly high proportion (>30%) of $T1/ST2^+$ T cells (18).

Surprisingly, a clear Th1 response was detectable at 1 and 4 weeks postinfection in both lymphoid organs, despite the pres-

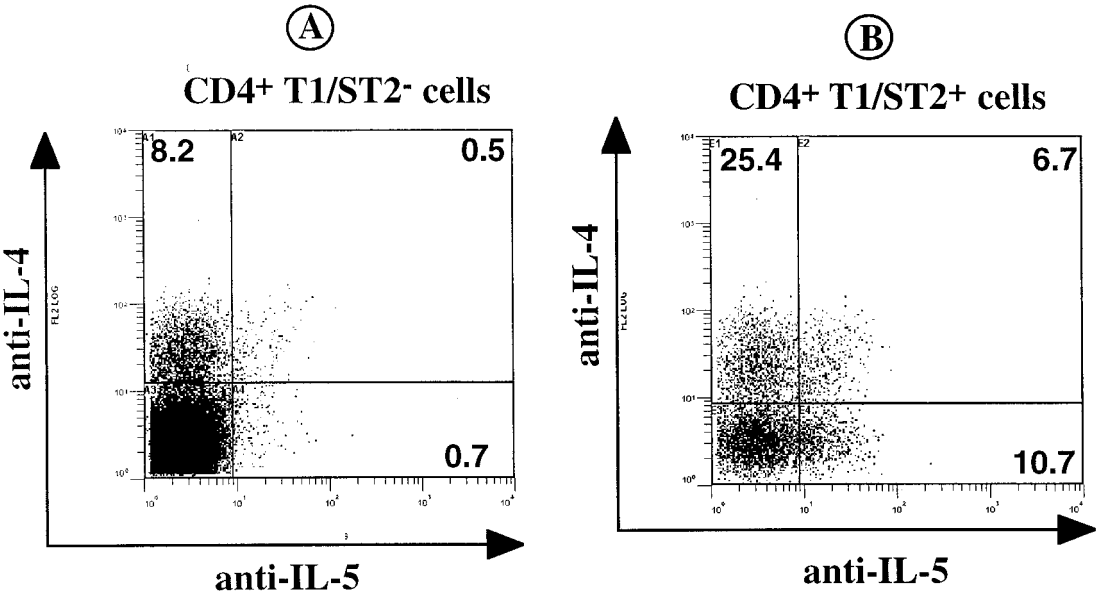


FIG. 3. Expression and coexpression of IL-4 and IL-5 in $CD4^+$ $T1/ST2^+$ and in $CD4^+$ $T1/ST2^-$ T cells. BALB/c mice were infected with *L. major* parasites and, 1 week postinfection, the spleens were harvested and restimulated with antigen. After 6 days, a Ficoll gradient was performed, $CD4^+$ T cells were purified by MACS and restimulated with PMA and ionomycin in the presence of brefeldin A as described in Materials and Methods and labeled with anti-T1/ST2, anti-IL-4, and anti-IL-5. Gate A was set up on $T1/ST2^-$ T cells, and gate B was set up on $T1/ST2^+$ T cells. The frequencies of IL-4- and IL-5-expressing cells were determined by flow cytometry. The results of one out of three similar experiments are presented.

ence of a strong Th2 response. Indeed, the frequency of IFN-γ-expressing $CD4^+$ T cells and the expression of T-bet are clearly demonstrated (Fig. 1, 4, and 5). However, since disease progression is not controlled, the protective effect of IFN-γ is not sufficient or is counteracted by the strong Th2 response.

The intracellular cytokine profile of $CD4^+$ T cells from *L. major*-infected BALB/c mice is not widely used to distinguish Th1 and Th2 cells. Only one study characterized the cytokine pattern of $CD4^+$ T cells during the course of infection with *L. major* and described Th2 cells as "nonclassical" Th2 cells because no T cells doubly positive for IL-4 and IL-10 were detected ex vivo (29). It is likely that differences in the protocols used (directly ex vivo and after in vitro enrichment of antigen-specific cells) account for the differences in these results, as it is possible that the $CD4^+$ $IL\text{-}4^+$ $IL\text{-}10^+$ T cells were below the ex vivo detection limit (Table 2).

Importantly, our results also reveal that two distinct populations of type 2 cells were present in the lymphoid organs of *L. major*-infected BALB/c mice: a subpopulation of $CD4^+$ T cells negative for the expression of T1/ST2 expressed IL-4, IL-10, and GATA-3 and the $CD4^+$ $T1/ST2^+$ T cells also expressed IL-4, IL-10, and GATA-3 and, in addition, expressed IL-5 and T1/ST2. It is unlikely that in our model the expression of T1/ST2 on *L. major*-specific $CD4^+$ T cells is a sign of a later commitment to the Th2 phenotype as suggested by Meisel et al. (12), since restimulation of the lymphoid cells in the presence of selection pressure did not alter the frequency of $CD4^+$ T cells expressing the T1/ST2 molecule (9a) or the frequency of $CD4^+$ T cells expressing IL-4 (Fig. 1). The finding that two distinct populations of Th2 cells can be distinguished by the expression of T1/ST2 also shows that the T1/ST2 marker does not exclusively correlate with Th2 cells; indeed a population of $CD4^+$ T cells negative for the expression of T1/ST2 expressed IL-4 and IL-10 and excluded IFN-γ. Interestingly, other results suggest that these two distinct Th2 populations have different biological functions (P. Kropf, S. Herath, R. Klemenz, and I. Müller, submitted for publication): $CD4^+$ $T1/ST2^+$ T cells but not $CD4^+$ $T1/ST2^-$ T cells were shown to regulate IL-12 responsiveness. Taken together, these results suggest that T1/ST2 may be a marker for a Th2 population with regulatory functions.

A role for IL-5 during the course of infection with *L. major* has not yet been defined; however, in light of the results mentioned above, the possibility that it could have an important function in the early response to *L. major* parasites cannot be excluded. IL-5 and eosinophils have been shown to be involved in different models of Th2-mediated lung inflammatory responses (2, 11, 32). Treatment with an anti-T1/ST2 monoclonal antibody or a T1/Fc fusion protein reduced the eosinophils and the production of IL-5, thereby inhibiting the immunopathology (2, 11, 32). In addition, *S. mansoni*-infected $T1/ST2^{-/-}$ mice did not develop granulomas and the lymphocytes isolated from the lungs displayed a severely impaired production of antigen-specific IL-5 (31). Thus, these results indicate that $CD4^+$ $T1/ST2^+$ T cells and IL-5 are involved in inflammation. The results presented in Fig. 3 show that IL-5 was expressed exclusively by the $CD4^+$ $T1/ST2^+$ T cells; therefore it is possible that these cells are involved in inflammatory responses. Indeed, our results have shown that the highest frequency of $CD4^+$ $T1/ST2^+$ T cells was detectable at the site of pathology, in the footpads (9a). Therefore, it is possible that, at that site, IL-5 might contribute to the inflammatory responses eventually leading to ulceration of the footpads during the nonhealing form of *L. major* infection. Indeed, treatment of *L. major*-infected BALB/c mice with a polyclonal anti-T1/ST2 anti-se-

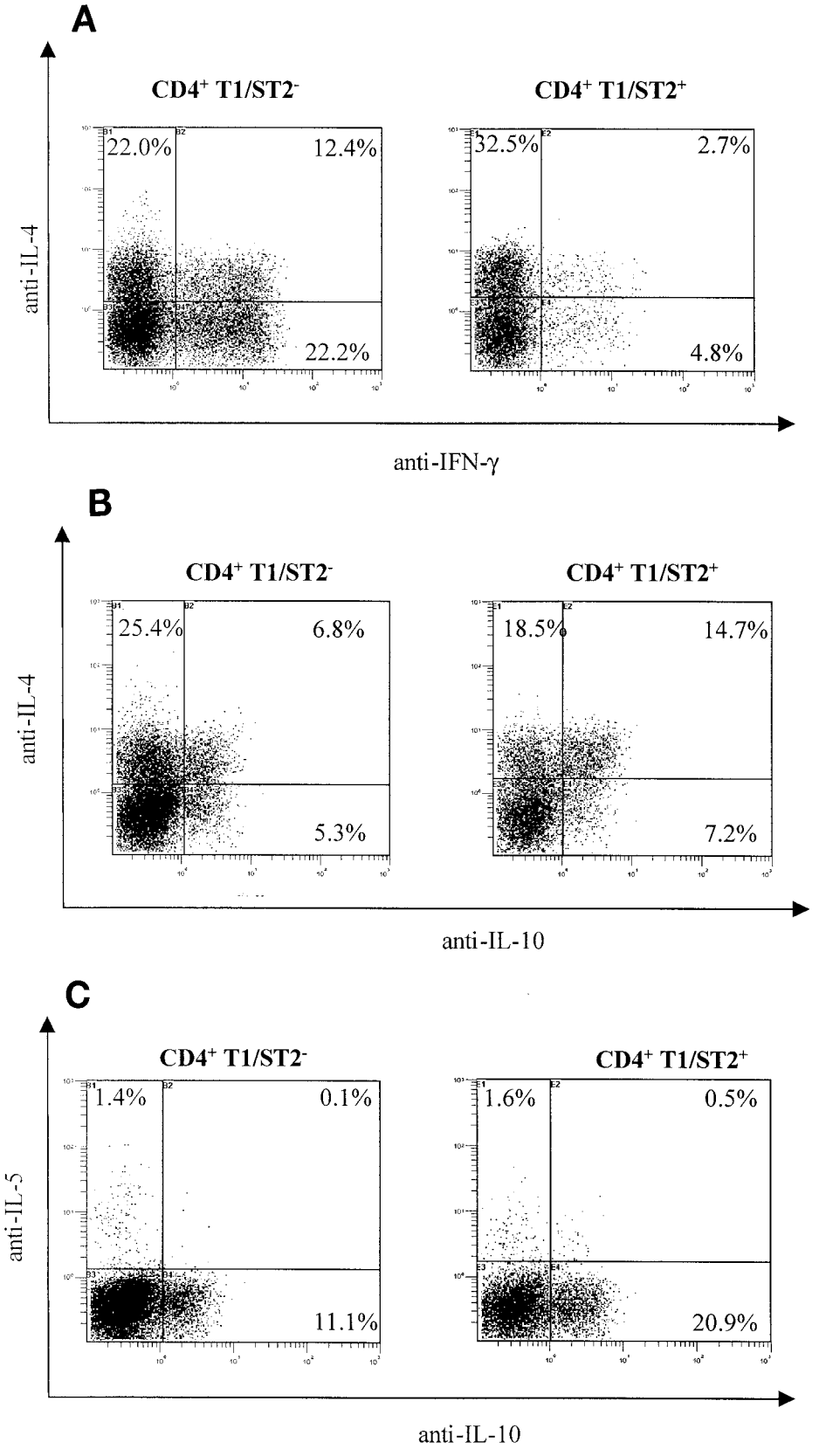


FIG. 4. Expression and coexpression of Th1 and Th2 cytokines in $CD4^+$ $T1/ST2^+$ and $CD4^+$ $T1/T2^-$ T cells. BALB/c mice were infected with *L. major* parasites and, 4 weeks postinfection, the draining lymph nodes were harvested and restimulated with antigen. After 6 days, a Ficoll gradient was performed, and $CD4^+$ T cells were purified by MACS and restimulated with PMA and ionomycin in the presence of brefeldin A as described in Materials and Methods and labeled with anti-T1/ST2, anti-IL-4, and anti-IFN-γ, anti-IL-4 and anti-IL-10, or anti-IL-10 and anti-IL-5. Gate A was set up on the $T1/ST2^-$ T cells, and gate B was set up on the $T1/ST2^+$ T cells (Fig. 1A). The frequencies of cytokine-expressing cells were determined by flow cytometry. The results of one out of three similar experiments are presented.

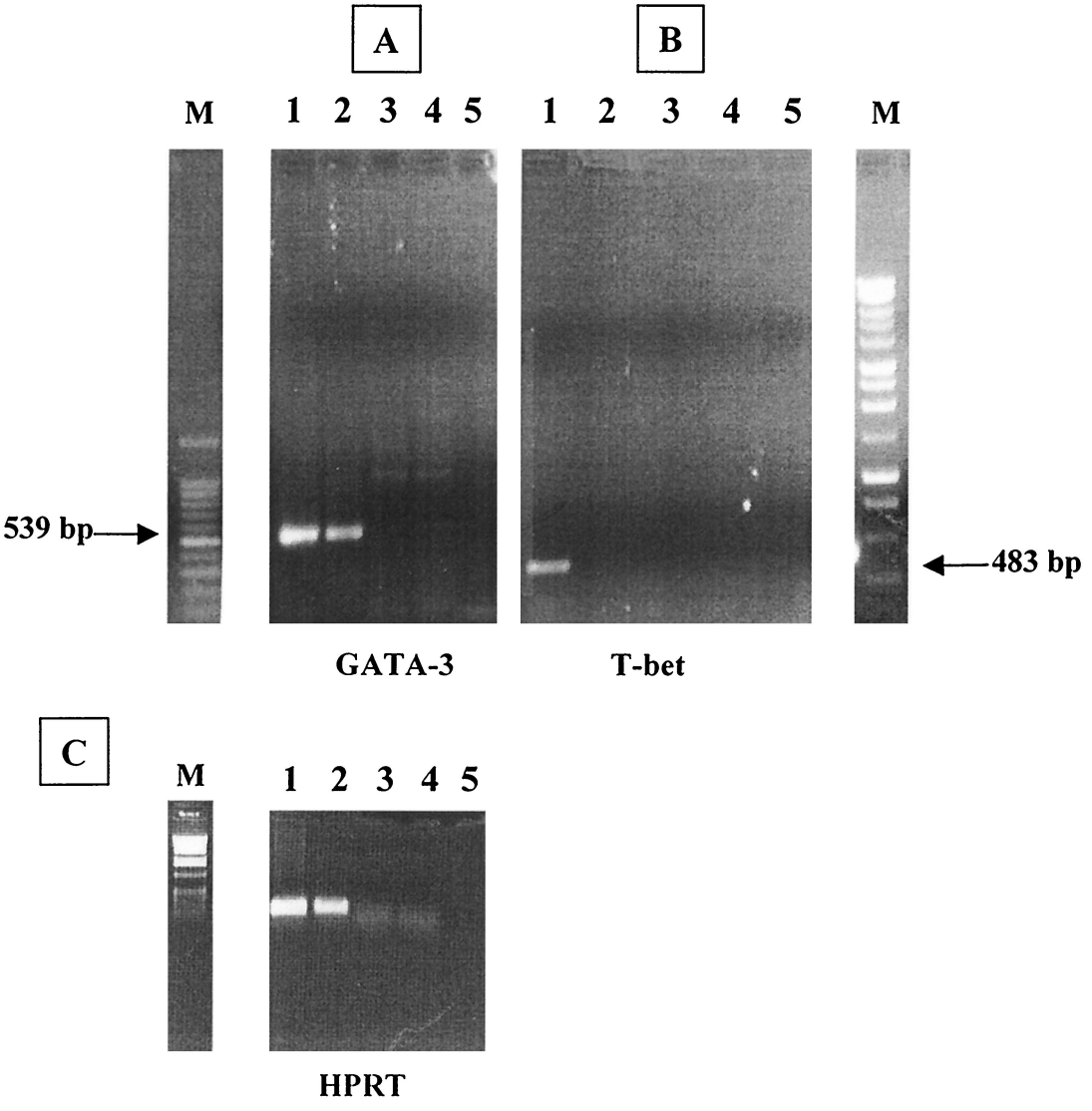


FIG. 5. Expression of GATA-3 and T-bet in $CD4^+$ $T1/ST2^+$ T cells and $CD4^+$ $T1/ST2^-$ T cells. The expression of GATA-3 (A), T-bet (B), and HPRT (C) in $CD4^+$ $T1/ST2^+$ T cells and $CD4^+$ $T1/ST2^-$ T cells was determined by reverse transcriptase PCR as described in Materials and Methods. Lanes 1, $CD4^+$ $T1/ST2^-$ T cells; lanes 2, $CD4^+$ $T1/ST2^+$ T cells; lanes 3 to 5, negative controls run with the RNA template but without the reverse transcription step with $CD4^+$ $T1/ST2^-$ (lanes 3) and $CD4^+$ $T1/ST2^+$ (lanes 4) T cells and run with all PCR reagents but without the template (lanes 5).

rum significantly reduced the lesion size and also reduced the levels of antigen-specific IL-5 (34).

The presence of two distinct subsets of Th2 cells might help to reconcile the controversial results obtained with $T1/ST2^{-/-}$ mice and explain why these mice did not display an impaired development of Th2 responses. Indeed, two independent studies have shown that $T1/ST2^{-/-}$ mice display a normal Th2 response after infection with *Nippostrongylus brasiliensis* (5, 28) and that the worms are expelled similarly in the wild-type and in the $T1/ST2^{-/-}$ mice. Therefore, it is possible that the population of $CD4^+$ $T1/ST2^-$ Th2 cells is accountable for the generation of the Th2 response leading to the expulsion of the worms.

In summary, the results presented in this study show that two distinct subsets of Th2 cells coexist in BALB/c mice infected with *L. major*. These two subsets can be distinguished by the expression of the T1/ST2 molecule and, early during the course of infection, by the expression of IL-5.

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