Infected site-restricted Foxp3$^+$ natural regulatory T cells are specific for microbial antigens

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Natural regulatory T (T reg) cells are involved in control of the immune response, including response to pathogens. Previous work has demonstrated that the repertoire of natural T reg cells may be biased toward self-antigen recognition. Whether they also recognize foreign antigens and how this recognition contributes to their function remain unknown. Our studies addressed the antigenic specificity of natural T reg cells that accumulate at sites of chronic infection with *Leishmania major* in mice. Our results support the idea that natural T reg cells are able to respond specifically to foreign antigens in that they strongly proliferate in response to Leishmania-infected dendritic cells, they maintain Foxp3 expression, and Leishmania-specific T reg cell lines can be generated from infected mice. Surprisingly, the majority of natural T reg cells at the infected site are Leishmania specific. Further, we showed that parasite-specific natural T reg cells are restricted to sites of infection and that their survival is strictly dependent on parasite persistence.

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Abbreviations used: B/6, C57BL/6; BMDC, bone marrow–derived DC; T reg, regulatory T.

Regulatory cells, by virtue of their capacity to control the vigor of immune responses, are essential to the maintenance of host homeostasis (1, 2). Several types of CD4$^+$ regulatory T (T reg) cells exist, some of which are induced in response to infectious challenge (so-called inducible T reg cells or antigen-specific T reg cells), and some of which are considered naturally occurring regulators (natural T reg cells; reference 3). Inducible T reg cells, such as Tr1 or T_H3 cells, can develop from conventional CD4$^+$ T cells that are exposed to specific stimulatory conditions (3–5). Natural T reg cells, however, arise during the normal process of maturation in the thymus and express a specific set of cell surface markers (for review see references 1 and 6). Recent studies also indicate that a unique transcription factor, Foxp3, is required for generation of natural T reg cells and that this represents, to date, their most specific marker (6). Natural T reg cells play a central role in the control of autoimmunity, a function associated with their capacity to recognize self-antigen. Whether they also recognize foreign antigens and the extent of their repertoire for such antigens remain unknown.

We and others have shown that natural T reg cells also play a critical role in the outcome of microbial infections (7). Natural T reg cells help limit collateral tissue damage caused by vigorous antimicrobial immune responses. These cells can also limit the magnitude of effector responses that result in failure to adequately control infection. Furthermore, there is clear evidence that the efficiency of vaccines can also be hampered by the presence of natural T reg cells. Thus, strategies to manipulate natural T reg cell function and numbers have high therapeutic potential. To develop rational strategies, there is an urgent need to better characterize the function of natural T reg cells during infections. In particular, one central question that must be addressed is whether the regulatory function of natural T reg cells is associated with their capacity to recognize foreign antigens or is the consequence of bystander

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activation through self-antigen recognition. In this report, we have specifically addressed the nature of the antigens recognized by naturally occurring T reg cells during infection.

Using an intradermal low-dose model of Leishmania infection, we have previously shown that natural T reg cells are essential for the development and maintenance of cutaneous infection with *Leishmania major* in resistant C57BL/6 (B/6) mice (8). Natural T reg cells rapidly accumulate at sites of *L. major* infection, favoring early parasite expansion. After effector-mediated control of the infection, natural T reg cells accumulate again in the infected tissue to control effector T cell functions, thus enabling the establishment of parasite persistence (8).

In this report, we address the capacity of natural T reg cells that accumulate at sites of Leishmania infection to recognize parasitic antigens. Our work supports the idea that natural T reg cells can recognize microbial antigens and that this recognition is necessary for their function and survival.

RESULTS

The majority of CD4$^+$CD25^{hi} T cells from the regional LN of chronically infected mice proliferate in response to *L. major*–infected DCs

We have previously shown that naturally occurring T reg cells accumulate at dermal sites of Leishmania infection and produce IL-10 in response to *L. major*–infected DCs (8). Such cytokine release did not occur in response to nonspecific signals, strongly supporting the idea that these cells were responding to Leishmania antigens (8). To formally assess the antigenic specificity of natural T reg cells during infection, we needed to obtain a population of T reg cells that we could expand in vitro. Because dermal T cells failed to proliferate in vitro under our experimental conditions (not depicted), we addressed the capacity of CD4$^+$CD25^{hi} T cells from the LN draining the chronically infected site (retromaxillar) to respond to Leishmania antigen. At this stage of infection (after wk 12), a small number of parasites was found in the draining LN (800 ± 200 parasites per LN; not depicted and reference 9), and no pathology was detectable at the primary site of infection. Purified CD4$^+$CD25^{hi} T cells were evaluated for their capacity to proliferate in response to *L. major*–infected DCs. Surprisingly, a large number of CD4$^+$CD25^{hi} T cells proliferated when exposed to *L. major*–infected bone marrow–derived DCs (BMDCs), as 60–85% of CD4$^+$CD25^{hi} cells have reduced CFSE intensity (Fig. 1). These cells have undergone up to four cycles of cell divisions in 3 d (Fig. 1). When T reg cells were stimulated by *L. major*–infected DCs or anti-CD3, the proliferation profile was not constituted by discreet populations. This is likely to be explained by the high variability in size of T reg cells from infected sites that is associated with different levels of CFSE content. In addition, polyclonal T reg cells were stimulated with *L. major*–infected DCs, which will present various antigens in a nonsynchronal manner. The absolute number of CD4$^+$CD25$^+$ T cells was also increased after incubation with *L. major*–infected DCs, ranging from a 2.9- to 10.2-fold increase compared with exposure to noninfected DCs (Table S1, available at http://www.jem.org/cgi/content/full/jem.20052056/DC1). Thus, in chronically infected mice, the majority of the CD4$^+$CD25^{hi} T cells from the LN draining the chronically infected site expanded when exposed to *L. major*–infected DCs.

CD4$^+$CD25^{hi} T cells that proliferate in response to *L. major*–infected DCs are natural T reg cells

Expression of CD25 is not restricted to natural T reg cells. To reduce the possibility of contamination by recently activated effector CD25$^+$ T cells, CD4$^+$CD25^{hi} T cells were purified from chronically infected mice. We then needed to confirm that the CD25^{hi} cells were natural T reg cells. We have previously shown, using T cell–reconstituted RAG$^{-/-}$ mice, that the majority of cells that expressed CD25^{hi} in the *L. major*–infected dermis originated form naturally occurring

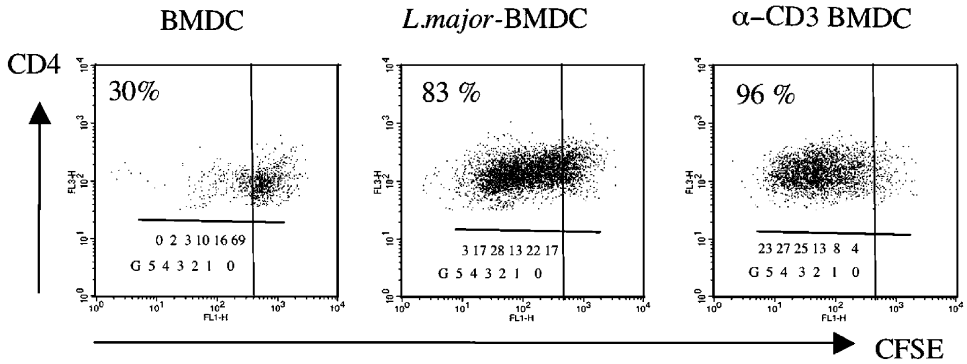

Figure 1. CD4$^+$CD25^{hi} T cells from the draining LNs of chronically infected mice proliferate in response to *L. major*–infected DCs. CD4$^+$CD25^{hi} T cells were sorted from the draining LNs of B/6 mice infected in the ear dermis with 10^3 *L. major* metacyclic promastigotes for 12 wk. 5 × 10^4 CFSE-labeled T cells were cultured for 3 d in 200 μl culture medium per well of a 96-well U-bottom plate with 2 × 10^5 BMDCs (with or without 0.5 μg/ml anti-CD3) or 2 × 10^5 *L. major*–infected BMDCs. Cells were analyzed by flow cytometry, and CD4$^+$TCR-β$^+$ gated cells are shown. The percentage of cells that divided is indicated. The numbers represent the frequency of events in each generation (G) based on ModFit analysis. Results are representative of seven independent experiments.

T reg cells (8). To determine whether this was also true in the regional LN during chronic infection, we purified $CD4^+CD25^{hi}$ cells from congenic ($Ly5.1^+$) naive mice and coinjected them with $CD4^+CD25^-$ cells from WT background (Ly5.2) at the time of *L. major* infection. At 12 wk after infection and transfer, the LN cells were collected and their phenotype was analyzed by flow cytometry. The cells that expressed $CD25^{hi}$ originated from naturally occurring T reg cells because 93% of them expressed the congenic marker Ly5.1 (Fig. 2 A). Furthermore, 99% of the transferred $Ly5.1^+$ cells ($CD25^+$) remained $Foxp3^+$ in the regional LN (Fig. 2 B) and dermis (not depicted). In contrast, only 1% of the transferred Ly5.2 $CD4^+CD25^-$ cells expressed Foxp3 in the LN, suggesting that no conversion from $Foxp3^-$cells has occurred in this compartment (Fig. 2 B). Because this observation was made in a lymphopenic environment, we needed to confirm that the cells undergoing proliferation in response to infected DCs in WT animals were natural T reg cells. We assessed the expression of the natural T reg cell–specific marker Foxp3 (6). The expression of this marker was evaluated on the $CD25^-$, $CD25^{lo}$, and $CD25^{hi}$ subsets of $CD4^+$ T cells from the regional LNs of chronically infected WT mice. As shown in Fig. 2 C, CD25 expression paralleled that of Foxp3, with 96% $CD25^{hi}$, 77% $CD25^{lo}$, and 2% $CD25^-$ expressing this transcription factor.

We next evaluated cytokine production by the three subsets of $CD4^+$ T cells purified from the regional LN in response to Leishmania antigen (Fig. 3). Analysis of antigen-experienced cells based on the expression of CD45RB showed that this compartment was also strongly enriched in $Foxp3^+$ cells (33% of $CD45RB^{lo}$ cells are $Foxp3^+$ compared with 2% for the total $CD4^+CD25^-$ T cell compartment; Fig. 2 C and Fig. S1, which is available at http://www.jem.org/cgi/content/full/jem.20052056/DC1). Similarly, the population enriched in effector–memory T cells ($CD62L^-$) was enriched in $Foxp3^+$ cells (Fig. S1). Thus, we cannot accurately determine the frequency of Leishmania-specific cells able to proliferate in the $CD4^+CD25^-$ T cell compartment. We do acknowledge that the total $CD4^+CD25^-$ does not represent the most appropriate compartment, as it also contains a large

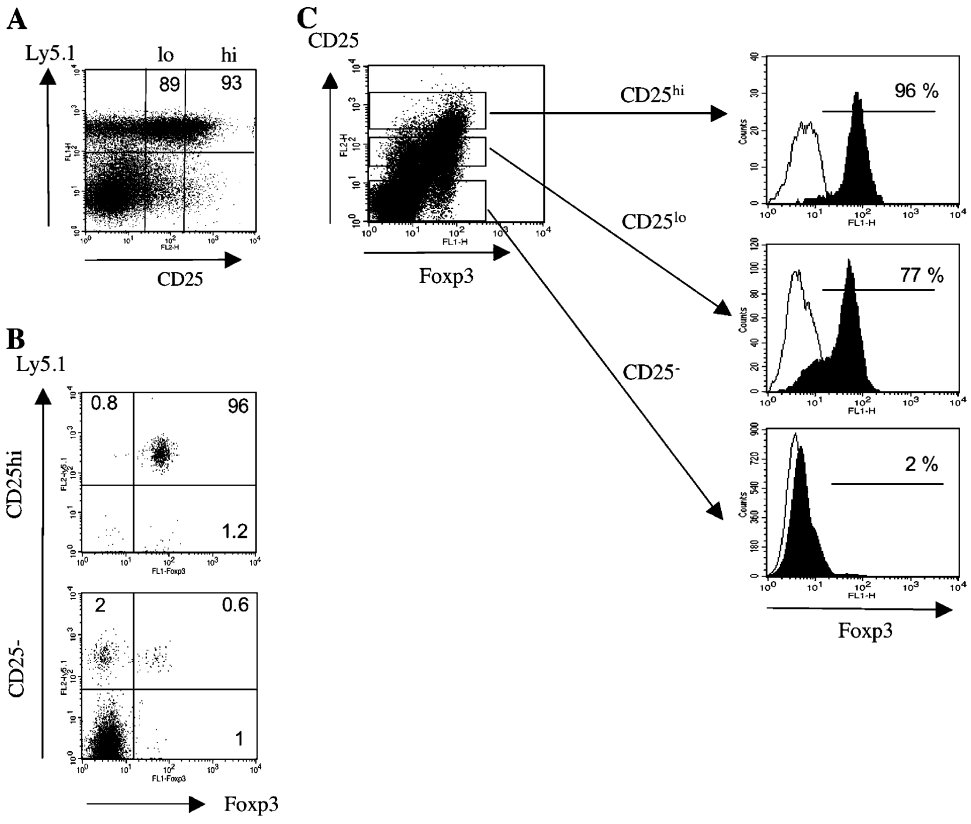

Figure 2. $CD4^+CD25^{hi}$ T cells from the draining LNs of chronically infected mice originate from naturally occurring T reg cells and express Foxp3. (A) $RAG2^{-/-}$ were reconstituted on the day of *L. major* infection with 3×10^4 $CD4^+CD25^{hi}$ $CD45.1^+$ T cells (Ly5.1) and 3×10^5 $CD4^+CD25^-CD45.2^+$ T cells (Ly5.2) from naive B6.SJL and B/6 mice, respectively. Values indicate the percentage of $CD45.1^+$ cells within the $CD25^{hi}$ and $CD25^{lo}$ T cell subsets (12 wk after infection). (B) The expression of Foxp3 was evaluated by flow cytometry. Events were gated on CD4 and CD25 expression as indicated. Values indicate the percentage of events in the indicated quadrant. (C) WT B/6 mice were inoculated in the ear dermis with 10^3 *L. major* metacyclic promastigotes. At 12 wk after infection, the draining LNs were collected and cells were analyzed by flow cytometry. Results are shown for $CD4^+TCR\text{-}\beta^+$ cells. Values indicate the percentage of $Foxp3^+$ cells (filled histograms) for each CD25 subset, as defined on the left panel, when compared with isotype control–stained cells (open histograms). Results are representative of three independent experiments.

proportion of naive cells; however, we used this population for the antigen-suppression assay because the contamination by Foxp3$^+$ cells was negligible (only 2% of the total cells; Fig. 2 C). The CD4$^+$CD25$^-$ T cells released the effector cytokines GM-CSF and IL-2 as well as low amount of IFN-γ but no IL-10 in response to *L. major*–infected BMDCs (Fig. 3). Transfer of 5 × 10^5 CD4$^+$CD25$^-$ T cells from the LNs of chronically infected mice conferred powerful immunity in naive mice infected with *L. major* (not depicted), demonstrating that this subset contained anti-Leishmania effector cells.

CD4$^+$CD25^{lo} T cells produce large amounts of IFN-γ (14 ± 0.13 ng/ml for 5 × 10^4 cells). This subset also produced IL-10 in response to the antigen. Thus, the level of Foxp3 (Fig. 2) and the cytokine profile of this subset suggested that this population could be either a mixture of effector and T reg cells or a distinct population of T reg cells. Although this subset may play an important role during infection, it remains heterogeneous. Therefore, we decided to focus our attention on CD4$^+$CD25^{hi} T cells.

CD4$^+$CD25^{hi} T cells produce IL-10 in response to *L. major* antigen (98 ± 10 pg/ml for 5 × 10^4 cells). This subset also produced small amounts of IFN-γ but no IL-2 or GM-CSF. Furthermore, the transfer of 5 × 10^5 CD4$^+$CD25^{hi} T cells from the LN into chronically infected mice triggered disease reactivation (not depicted and reference 10). We next addressed the suppressive function of the CD4$^+$CD25^{hi} subset in polyclonal and antigen-specific stimulation assays. CD4$^+$CD25^{hi} T cells from infected mice displayed comparable suppressive functions to CD4$^+$CD25^{hi} T cells purified from naive mice on CD4$^+$CD25$^-$ T cells activated with anti-CD3 in the presence of BMDCs (not depicted). More importantly, this subset inhibited release of IL-2 and GM-CSF by CD4$^+$CD25$^-$ T cells by 92 and 79%, respectively, in an antigen-dependent manner (Fig. 3). Of interest, the production of IL-10 by natural T reg cells was significantly increased (98 ± 10 to 338 ± 14 pg/ml; $P < 0.0001$) in the antigen-specific suppression assays.

Thus, based on (a) our transfer experiments showing that CD4$^+$CD25^{hi} T cells originated from naive naturally occurring T reg cells, (b) the level of Foxp3 expression of CD4$^+$CD25^{hi} T cells from WT infected mice, (c) their cytokine profile, and (d) their suppressive function, we concluded that the CD4$^+$CD25^{hi} T cells from the LN connected to the primary site were natural T reg cells. Thus, this population constitutes a valid source to address the specificity of natural T reg cells. Our results demonstrate that the majority of natural T reg cell compartments of the LN associated to the site proliferate in response to Leishmania-infected DCs.

CD4$^+$CD25$^+$Foxp3$^+$ T cells that have divided in response to *L. major*–infected DCs maintain Foxp3 expression after division

As previously shown, 95% of the CD4$^+$CD25^{hi} T cells from the draining LN are Foxp3$^+$; nevertheless, we needed to confirm that the cells that had undergone division in vitro remained Foxp3$^+$. T cell subsets were evaluated for expression of this transcription factor after division. Among the CD4$^+$CD25^{hi}

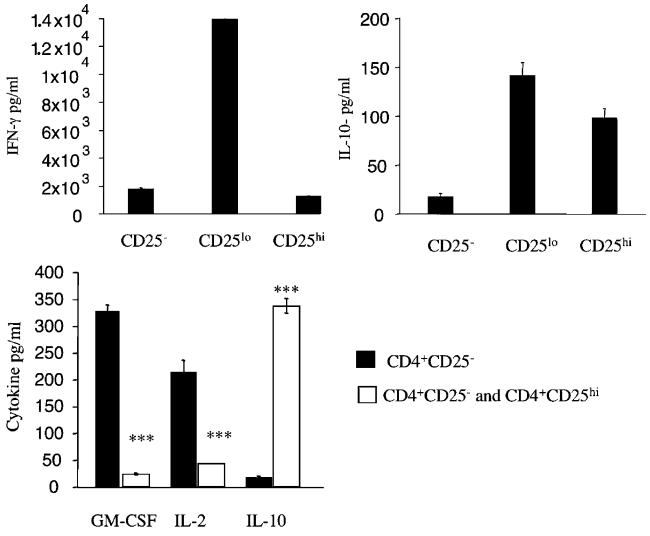

Figure 3. CD4$^+$CD25^{hi} T cells from the draining LNs of chronically infected mice suppress CD4$^+$CD25$^-$ T cell cytokine production in an antigen-dependent manner. CD4$^+$ T cell populations were sorted from the draining LNs of B/6 mice infected in the ear dermis with 10^3 *L. major* metacyclic promastigotes for 12 wk. Top histogram: 5 × 10^4 CD4$^+$ T cells from each CD25 subset were stimulated with 2 × 10^5 *L. major*–infected BMDCs for 4 d in 200 μl culture medium per well of a 96-well U-bottom plate. Secreted cytokines (IFN-γ and IL-10) were quantitated by multiplex assay. Data are expressed as mean ± SD (pg/ml). Bottom histogram: 5 × 10^4 CD4$^+$CD25$^-$ T cells were stimulated (alone [filled bars] or cocultured with 5 × 10^4 CD25^{hi} T cells [empty bars]). Data are representative of three independent experiments. Asterisks represent statistically significant differences between indicated groups (***, $P < 0.0001$).

T cells undergoing proliferation, 96% were Foxp3$^+$ after proliferation. Of interest, exposure to *L. major*–infected DCs is required for sustained Foxp3 expression because only 72% of the CD25^{hi} T cells remained positive for Foxp3 (with a mean fluorescence intensity of 58) with noninfected BMDCs versus 90% maintaining Foxp3 expression when exposed to infected DCs (mean fluorescence intensity of 129). Alternatively, this decrease may reflect a differential survival of the cells under these two culture conditions. CD4$^+$CD25^{lo} T cells proliferate in response to infected DCs, and this proliferation was equally distributed between Foxp3$^+$ and Foxp3$^-$ cells (Fig. 4). In contrast, CD25$^-$ Foxp3$^-$ cells remained negative for Foxp3 after incubation with infected DCs. Exposure to TGF-β in the presence of anti-CD3 and anti-CD28 has been shown to induce Foxp3 expression on CD4$^+$CD25$^-$Foxp3$^-$ cells (11). To determine whether such stimuli could convert the CD25$^-$ subset or enhance Foxp3 expression in the other populations in our model, we incubated the cells with TGF-β and DCs that were infected or not. No differences in the percentage or intensity of Foxp3 were observed for the three populations (Fig. 4 and not depicted).

CD4$^+$CD25$^+$Foxp3$^+$ T cells divide in response to *L. major* in an antigen-dependent manner

We next addressed the possibility that the proliferation of CD4$^+$CD25$^+$Foxp3$^+$ T cells could be the consequence of

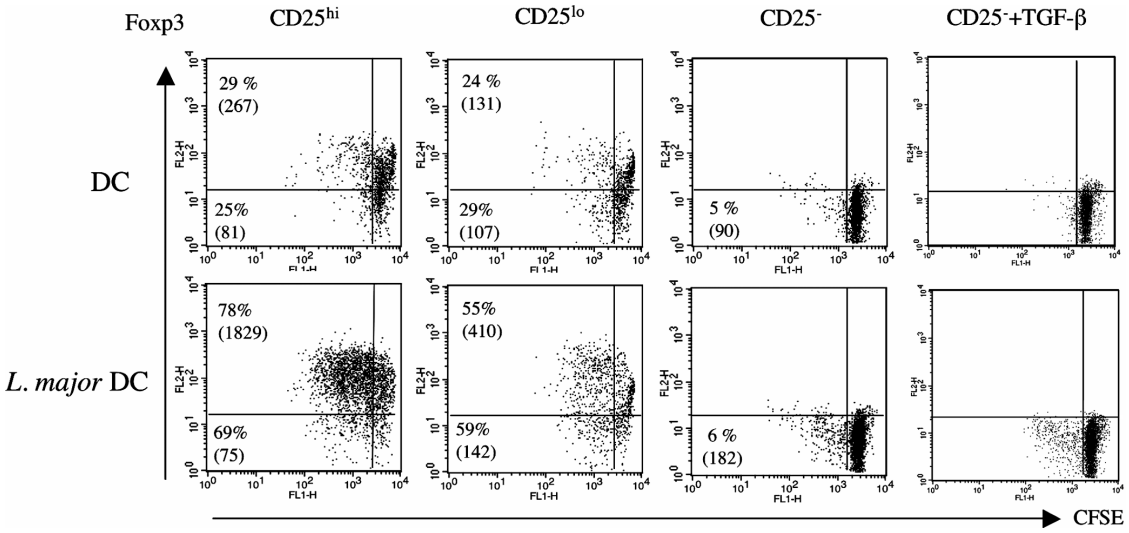

Figure 4. CD4$^+$CD25^{hi} T cells that proliferate in response to *L. major* antigens maintain Foxp3 expression. CD4$^+$CD25^{hi}, CD4$^+$CD25^{lo}, and CD4$^+$CD25$^-$ T cells were sorted from the draining LNs of B/6 mice infected in the ear dermis with 10^3 *L. major* metacyclic promastigotes for 12 wk. 5×10^4 CFSE-labeled T cells from each CD25 subset were stimulated for 3 d with 2×10^5 *L. major*–infected BMDCs (bottom) or noninfected BMDCs (top). CD4$^+$CD25$^-$ T cells were cultured with or without 2 ng/ml recombinant TGF-β1. Cells were analyzed by flow cytometry. CD4$^+$ TCR-β$^+$ gated cells are shown. The percentage of cells that divided and are Foxp3$^+$ is indicated. Numbers in parenthesis are the absolute number of cells in the indicated gate. Results are representative of three independent experiments.

microbial products and/or unspecific activation. Purified CD4$^+$CD25^{hi} T cells from infected mice were exposed to BMDCs that were generated from WT or MHC class II$^{-/-}$ mice. In the absence of MHC class II molecules, CD4$^+$CD25$^+$ T cell proliferation was abolished (Fig. 5).

Because *L. major* has been shown to activate DCs (12), we needed to exclude the possibility that T reg cells from chronically infected mice became more responsive to activated DCs. Thus, CD4$^+$CD25^{hi} T cells from chronically infected mice were exposed to BMDCs previously activated with LPS alone or LPS with anti-CD40 agonist (Table I). Such treatment failed to induce the proliferation of T reg cells as well as IL-10 release (Table I and not depicted). In addition, no proliferation or cytokine production was observed when T reg cells were purified from retromaxillar LNs of naive mice and exposed to *L. major*–infected BMDCs (Table I). Collectively, these results suggest that natural T reg cells from chronically infected mice are able to respond to *L. major*–infected DCs in an antigen-dependent manner.

Leishmania-specific natural T reg cells are restricted to sites of infection

After clinical cure, a small number of *L. major* persists at sites of infection and in the regional LN (9). The distribution of T reg cells that respond to microbial antigens during infection remains unexplored. For instance, are these cells localized to the sites of parasite persistence or disseminated in distal organs as memory T cell populations? We evaluated the distribution of T reg cells that respond to *L. major* in various compartments. Only T reg cells that were purified from the LN draining the primary site of infection responded to infected BMDCs by proliferating (Fig. 6 A) and releasing IL-10 (Fig. 6 B). No proliferation or IL-10 production was detected in distal sites, such as the spleen or cervical or mesenteric LN (Fig. 6, A and B). This is in contrast

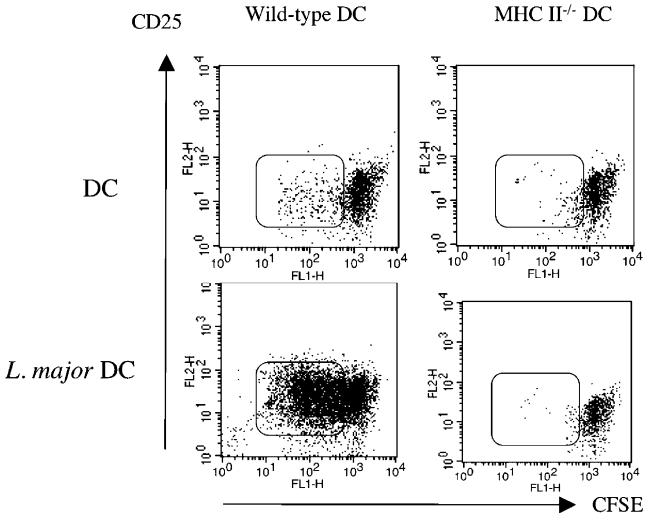

Figure 5. CD4$^+$CD25^{hi} T cell proliferation in response to *L. major* antigens is MHC class II restricted. CD4$^+$CD25^{hi} T cells were sorted from the draining LNs of B/6 mice infected in the ear dermis with 10^3 *L. major* metacyclic promastigotes for 5 mo. 5×10^4 CFSE-labeled T cells were stimulated for 5 d with 2×10^5 *L. major*–infected BMDCs (bottom) or noninfected BMDCs (top) generated from WT or MHC class II$^{-/-}$ mice. Cells were analyzed by flow cytometry. CD4$^+$TCR-β$^+$ gated cells are shown. Results are representative of two independent experiments.

Table I. Antigen-specific stimulation of $CD4^+CD25^+$ T cells by infected BMDCs

	$CD4^+CD25^{hi}$ T cells from: [a]					
	Chronically infected mice				Naive mice	
Number of cell divisions	BMDCs	Infected BMDCs	LPS-treated BMDCs	Anti-CD40 and LPS-treated BMDCs	BMDCs	Infected BMDCs
0	93.2	32.9	82.1	86.3	74.7	68.5
1	4.4	33.7	15.5	7.4	18.9	19.5
2	1.2	14.3	2.2	3.8	4.5	6.6
3	1.2	15.3	0.2	1.6	1.1	3.4
4	0.0	3.8	0.0	0.9	0.9	1.9

Data are representative of at least two independent experiments.
[a]$CD4^+CD25^{hi}$ T cells were sorted from the draining LNs of B/6 mice infected in the ear dermis with 10^3 *L. major* metacyclic promastigotes for 5 mo or from the same LNs of naive mice. 5×10^4 CFSE-labeled $CD4^+CD25^+$ T cells and 5×10^5 BMDCs were cultured for 3 d. BMDCs were either untreated or previously incubated overnight with *L. major* metacyclic promastigotes or stimulated with 50 ng/ml LPS (alone or with 1 μg/ml anti-CD40). Cells were analyzed by flow cytometry, and cell divisions were analyzed on the gated $CD4^+TCR\text{-}\beta^+$ cells with ModFit software.

to $CD4^+CD25^-$ T cells, which can produce IFN-γ when purified from the regional LN but also when purified from the spleen. Thus, T reg cells that respond to parasitic antigen do not disseminate in organs in which the parasite is absent.

To confirm that T reg cells have enhanced proliferation in vivo at sites of infection, we treated the mice with BrdU for 4 d (Fig. 6 C). At this point, BrdU incorporation in $CD4^+CD25^{hi}$ T cells from various compartments was evaluated by flow cytometry. Between 9 and 12% of

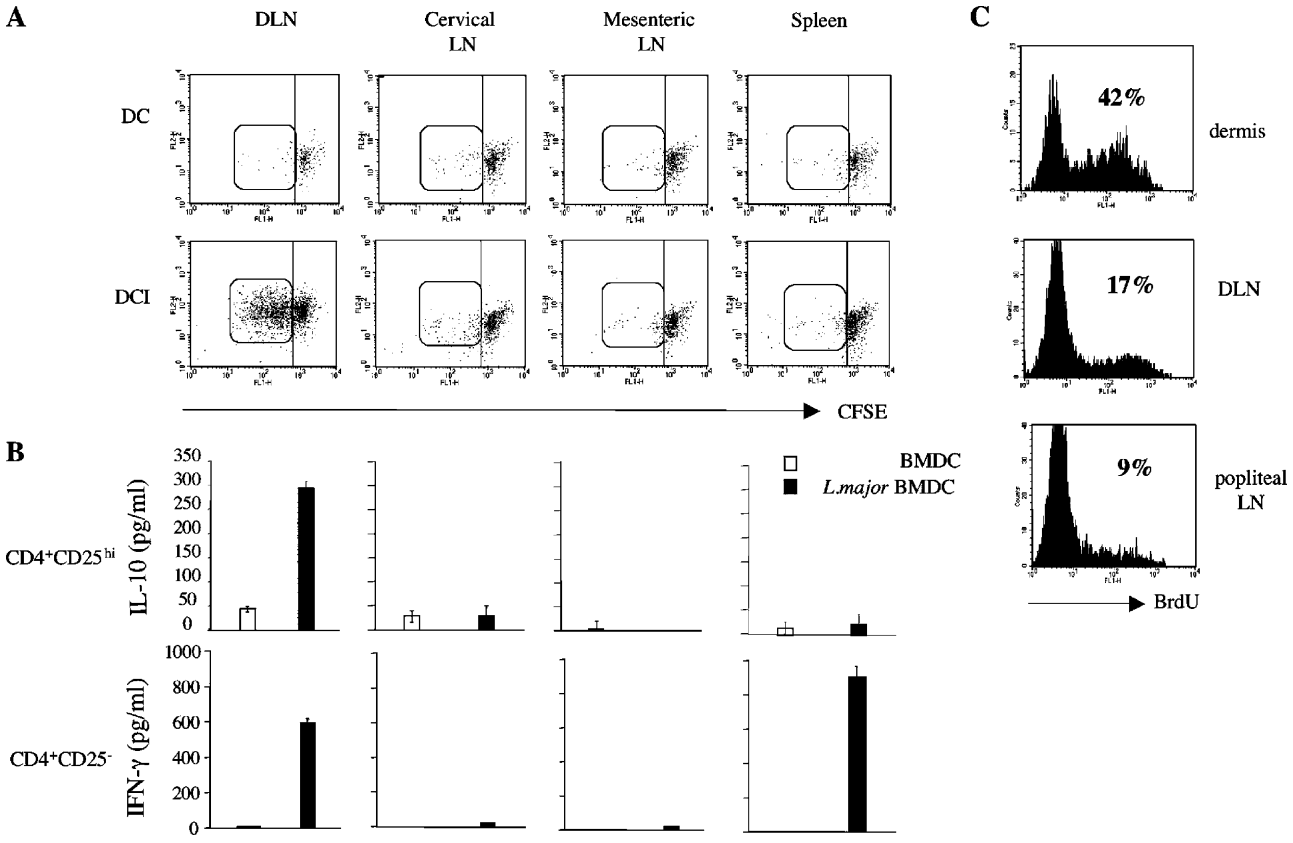

Figure 6. Leishmania-specific $CD4^+CD25^{hi}$ T cells are restricted to sites of parasite persistence. B/6 mice were infected in the ear dermis with 10^3 *L. major* metacyclic promastigotes for 12 wk. (A) $CD4^+CD25^{hi}$ and $CD4^+CD25^-$ T cells were sorted from the draining LN (DLN), cervical LN, mesenteric LN, or spleen and labeled with CFSE. 5×10^4 T cells were stimulated for 4 d with 2×10^5 *L. major*–infected BMDCs (bottom) or noninfected BMDCs (top). Cells were analyzed by flow cytometry. $CD4^+TCR\text{-}\beta^+$ gated cells are shown. (B) T cells were stimulated as indicated in A, and secreted cytokines were quantitated by ELISA. Data are representative of two independent experiments. (C) Mice were treated with 1 mg BrdU/day, i.p., for 4 d. Cells from the dermis, draining LN, and popliteal LN were analyzed the next day by flow cytometry. $CD4^+CD25^{hi}TCR\text{-}\beta^+$ gated cells are shown. Values indicate the percentage of $BrdU^+$ cells. Data are representative of three distinct experiments.

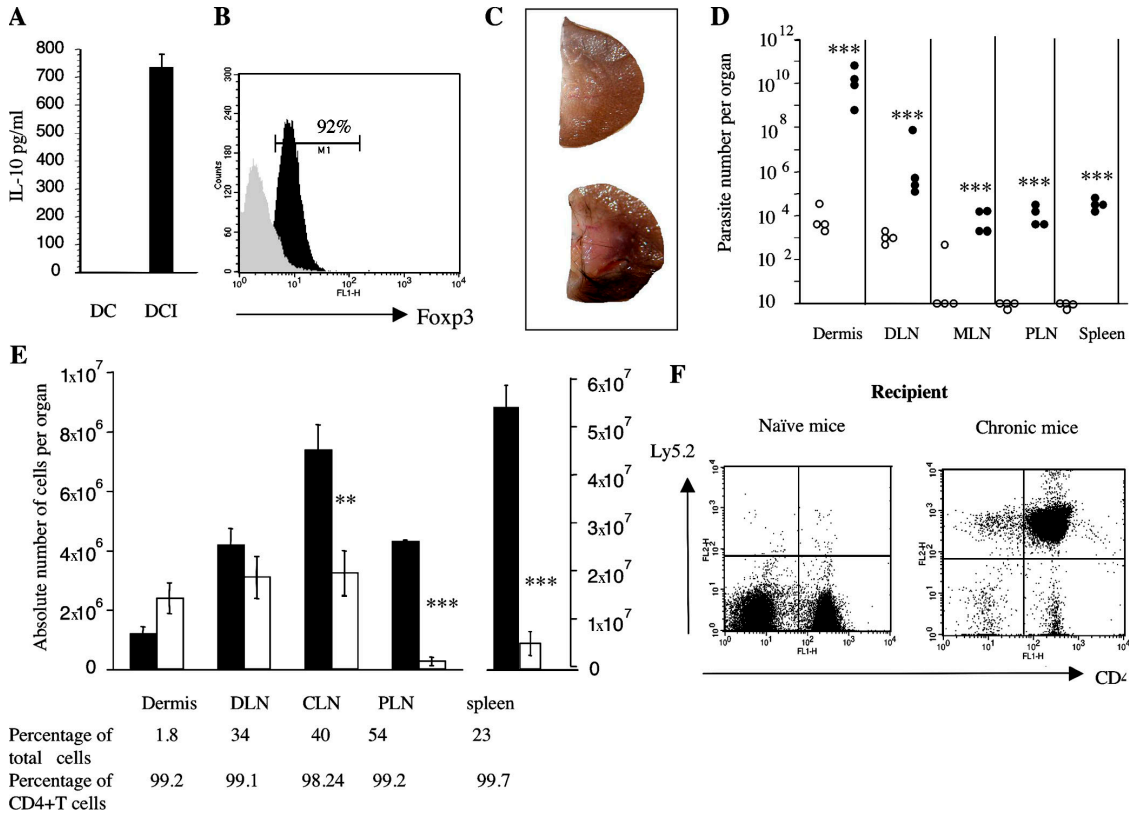

Figure 7. T reg cell lines specific for parasitic antigen maintain their regulatory function in vitro and in vivo. $CD4^+CD25^{hi}$ T cells were sorted from the draining LNs of B/6 mice infected in the ear dermis with 10^3 *L. major* metacyclic promastigotes for 4 mo. T cells were expanded weekly with irradiated *L. major*–infected BMDCs in the presence of 10 ng/ml IL-2 and irradiated spleen cells. (A) Expanded T cells were restimulated as described in Fig. 5. Secreted IL-10 was quantitated by ELISA. Results are representative of two independent assays of two independently generated T cell lines. (B) Foxp3 expression was evaluated by flow cytometry analysis of T cell lines. (C–F) 5×10^5 expanded T cells were transferred into B6.SJL mice inoculated in the ear dermis with 10^3 *L. major* metacyclic promastigotes 12 wk earlier. Results are shown 2 wk after transfer. (C) Ear lesions are shown for transferred (bottom) or untreated (top) mice. (D) Parasites were quantitated in the ear dermis, draining LN (DLN), mesenteric LN (MLN), popliteal LN (PLN), and spleen of T cell–transferred (filled circles) or untreated (open circles) mice. Four mice were analyzed in each group. Asterisks represent statistically significant differences between indicated groups (***, $P < 0.0001$). (E) Total numbers of cells recovered per organ are indicated in the histogram for T cell–transferred (filled bars) and untreated (open bars) mice. These cells were analyzed by flow cytometry, and transferred $CD4^+CD45.2^+$ T cells were quantitated as percentages of total cells (top row) or $CD4^+$ T cells (bottom row). Asterisks represent statistically significant differences between indicated groups (**, $P < 0.001$; ***, $P < 0.0001$). (F) Transferred $CD4^+CD45.2^+$ T cells were detected by flow cytometry into in the draining LN 2 wk after transfer into 12-wk–infected (right) or noninfected (left) B6.SJL (CD45.1 haplotype) mice. Results are representative of two independent assays of two independently generated T cell lines.

$CD4^+CD25^{hi}$ T cells from popliteal LNs have incorporated BrdU (a number comparable to that obtained from naive mice; not depicted). In contrast, 39–43% of $CD4^+CD25^{hi}$ T cells from infected dermis and between 15 and 19% of $CD4^+CD25^{hi}$ T cells from draining LNs have incorporated BrdU during this period of time. Thus, natural T reg cells have enhanced proliferative capacity at sites of chronic infection compared with distal LNs in vivo. To define whether natural T reg cells also have enhanced apoptosis at site of infection, we labeled the cells with annexin V. When purified from the LN associated with the infected site, the proportion of $annexin^+$ cells in the $CD4^+CD25^{hi}$ T cells was two times higher than in the $CD4^+CD25^-$ T cells ($19 \pm 3.3\%$ vs. $9 \pm 2.3\%$, respectively; four mice per group; not depicted).

We have previously shown that parasite persistence was required for maintenance of effector–memory responses against *L. major* infection. To address whether antigen persistence was also required for maintenance of T reg cells that respond to *L. major*, we treated chronically infected mice with anti–IL-10 receptor. We have previously shown that such treatment leads to sterile cure. After 3 wk, no parasites were detectable in the dermis and regional LN of the treated mice. At this point, $CD4^+CD25^{hi}$ T cells purified from the regional LN failed to proliferate or produce cytokine in response to *L. major*–infected BMDCs (not depicted). Thus, natural T reg cells at the site of chronic infection are characterized by a high proliferation/cell death turnover and are rapidly eliminated in absence of Leishmania antigens.

Generation of natural T reg cell lines specific for parasitic antigens

To further address the antigenic specificity of natural T reg cells that accumulate at sites of parasitic infection, we developed cell lines from chronically infected B/6 mice. $CD4^+CD25^{hi}$ T cells from the draining LN were purified and incubated with irradiated infected BMDCs in the presence of low amounts of IL-2 and irradiated spleen cells. A total of six T reg cell lines were maintained for 1 yr. These cells divided extensively (by a factor of 10 to 20 per week) and maintained their capacity to produce large amounts of IL-10 in response to *L. major*–infected BMDCs (Fig. 7 A). More importantly, T reg cell lines maintained Foxp3 expression (between 90 and 98% positive for Foxp3; Fig. 7 B). Thus, these cells are phenotypically and functionally indistinguishable from freshly purified T reg cells. To assess their suppressive function in vivo, we transferred 5×10^5 cells derived from $CD4^+CD25^{hi}$ T cells from WT B/6 (Ly5.2) mice into chronically infected (12 wk after infection) congenic (Ly5.1) mice. As early as 1 wk after transfer, mice reactivated their lesions as indicated by increased swelling of the primary site of infection (not depicted). At 2 wk after transfer, the disease was dramatically reactivated, with enhanced swelling of the ear (Fig. 7 C) and a substantial increase in parasite numbers at the primary sites (3 log in the dermis and 1.5 log in the draining LN). A surprisingly high number of parasites also disseminated in other organs in transferred mice (Fig. 7 D). The presence of T reg cell lines was evaluated by their expression of the congenic marker. In transferred chronic mice, T reg cell lines constituted 23–54% of the total cells found in the lymphoid organ analyzed (Fig. 7, E and F). In addition, T reg cell lines represented between 98 and 99% of the total $CD4^+$ T cells found in each compartment analyzed. In contrast, no cells from T reg cell lines were detected 2 wk after transfer into naive mice (Fig. 7 F).

Because parasitic infection could create a milieu that favors T reg cell expansion in a nonspecific manner we transferred a *L. major*–derived T reg cell line into *Toxoplasma gondii*–infected mice. No T reg cell lines were detected in mice that were infected for 2 wk with *T. gondii* at 3 wk after transfer (not depicted). Thus, $Foxp3^+$ T reg cell lines could be generated from chronically infected mice and maintained their antigen-specific suppressive function in vivo. Furthermore, when fully activated, these cells dramatically out-competed endogenous T cells.

DISCUSSION

It is becoming clear that naturally occurring $CD4^+CD25^+$ T reg cells not only influence self-antigen–specific immune responses but also dampen foreign antigen-specific immunity. Whether this effect is associated with their capacity to recognize foreign antigens and the extent of their repertoire for such antigens remain unknown. Our initial observation using a model of chronic infection by Leishmania showed that natural T reg cells accumulate at sites of infection in which they control effector immune responses allowing for pathogen persistence (8). In this report, we formally addressed the capacity of natural T reg cells present at the site of infection to respond to parasitic antigens.

Our results strongly support the idea that natural T reg cells are able to respond to foreign antigens in that (a) they proliferate in response to infected DCs, (b) the cells that have undergone proliferation express and maintain Foxp3, and (c) T reg cell lines that maintain natural T reg cell characteristics can be generated from infected mice. Surprisingly, the majority of the natural T reg cell compartment of the LN associated with the infected site is composed of cells that respond to Leishmania antigens. Furthermore, we showed that a mechanism by which natural T reg cells may be compartmentalized in vivo could be through their dependence on parasite persistence. This report is, to our knowledge, the first demonstration that natural T reg cells can respond to foreign antigens during infection.

Natural T reg cells are believed to recognize a wide array of self-antigens. Based on the major role that these cells play in the control of autoimmune responses and the fact that natural T reg cells have a greater capacity to recognize self-antigen than $CD4^+CD25^-$ T cells (13), it has been suggested that self-antigens may be their only antigenic specificity; however, the capacity of natural T reg cells to respond to foreign antigens is supported by the observation that natural T reg cells have a polyclonal TCR repertoire (13). In addition, how do natural T reg cells become recruited and control such a large array of infections (7) if the only antigens they recognize are tissue-specific self-antigens? Such a hypothesis is compelling during the onset of acute disease because tissue damage is also associated with enhanced presentation of self-antigen, but most systems for which a role for T reg cells has been described produce chronic, mild, or nonaggressive infections. In these cases, the hypothesis of self-reactivity is hard to reconcile unless pathogens aimed at surviving for extended periods of time in their host have specifically evolved to possess antigen that would cross react with self. We chose to address the specificity of natural T reg cells at the time of chronicity because this phase is characterized by low or no pathology, minimizing the possibility of enhanced self-antigen presentation. In this report, we showed that $CD4^+CD25^{hi}$ T cells from the regional LN proliferate and produce IL-10 in response to Leishmania-infected DCs. Such a response is dependent on the antigen presentation, as it is abolished in the absence of MHC class II molecules. Furthermore, nonspecific activation such as LPS or anti-CD40 agonist, previously shown to enhance natural T reg cell function (14), failed to induce such proliferation. In addition, infected DCs did not induce the proliferation of naive T reg cells, eliminating the possibility that infection of the DCs enhanced self-antigen presentation.

Transcription factor Foxp3 is believed to be the most specific marker of natural T reg cells (6). Under steady-state conditions, $CD25^{hi}$ T cells coexpress Foxp3 (15), and its expression correlates with the suppressive activity of $CD4^+$ T cells regardless of their level of CD25 expression (6). In our experimental model, the $CD4^+CD25^{hi}$ T cells from the regional LN that respond in an MHC class II–dependent manner to Leishmania antigen are indistinguishable from the endogenous

pool of natural T reg cells in that they are homogenously positive for Foxp3, remain Foxp3 positive after proliferation, and display powerful suppressive functions. It was recently shown that in vitro exposure of $CD4^+CD25^-$ T cells from naive mice to anti-CD28 and anti-CD3 in the presence of TGF-β can induce de novo expression of Foxp3 (11). To exclude the possibility that such a pathway was also occurring in our model, we performed a transfer experiment with a highly purified population of T reg cells using congenic markers to trace their origin. Using this approach, we were able to show that the only cells that express a high level of CD25 in the regional LNs of chronically infected mice did indeed derive from the endogenous pool of natural T reg cells. Furthermore, no de novo expression of Foxp3 was observed on the transferred $CD4^+CD25^-$ T cell population in infected sites. This is further supported by recent observations, using Foxp3 reporter mice, showing that homeostatic proliferation or infection of mice with *Listeria monocytogenes* failed to induce expression of Foxp3 in $CD4^+CD25^-$ T cells (15). In addition, in vitro exposure of $CD4^+CD25^-$ T cells from infected mice to TGF-β in the presence of Leishmania antigen failed to induce expression of this marker. Other supporting evidence for the notion that natural T reg cells can recognize microbial antigen comes from studies on Schistosoma infection. Natural T reg cells taken from chronically infected mice can also produce IL-10 in response to parasite antigens (8, 16, 17). Some of the most convincing data come from human studies. In HIV- or hepatitis C virus–infected individuals, cells with the characteristics of natural T reg cells mediate suppression in an antigen-specific manner (18) and produce IL-10 in response to viral antigen (19–21). Our present observations strongly support the idea that, in the pool of natural T reg cells, some can recognize foreign antigens and that this recognition is an essential step in their expansion and regulatory function.

Our studies revealed an unexpected high number of antigen-specific natural T reg cells at sites of chronic infection. Up to 80% of the natural T reg cells from the draining LN are able to proliferate when exposed to infected DCs in vitro, suggesting that these cells constitute the majority of the regional natural T reg cell compartment. This dramatic proliferative capacity is confirmed by our BrdU experiments revealing that a greater number of T reg cells proliferate at sites of chronic infection compared with the periphery. Thus, far from being anergic, as in vitro experiments suggested, natural T reg cells proliferate vigorously when they encounter their specific microbial antigens. Previously published studies in TCR transgenic mice showed that antigen-specific natural T reg cells divide when appropriately presented with antigen (22, 23). Previous reports have also shown a strong accumulation of natural T reg cells at sites of HIV infection (24). The continual accumulation of natural T reg cells could have potentially detrimental consequences by reducing the repertoire of local natural T reg cells and through local bystander suppression. Such effects may contribute to the poorer control of cancer and reactivation of persistent infections that occur in elderly individuals (25).

The distribution of T reg cells that respond to microbial antigens during infection remains unexplored. We investigated the distribution of natural T reg cells that respond to parasite antigens at sites of parasite persistence as well as in distal organs in which no parasites were detected. We found that the presence of natural T reg cells that respond to parasitic antigen is restricted to the sites of infection. This is in contrast to effector IFN-γ–producer cells, which can be found in distal sites. If natural T reg cells maintain strong proliferative capacity in response to the antigen, this expansion has to be controlled to prevent systemic immunosuppression. We have previously shown that parasite persistence was required for long-term maintenance of immunity (8). Similarly, T reg cells that respond to the parasite can no longer be detected when the mice are treated to achieve sterile cure. Furthermore, the percentage of T reg cells undergoing apoptosis was twice as high in the regional LN as that for $CD4^+CD25^-$ T cells. These results suggest that one mechanism by which the strong proliferative capacity of natural T reg cells could be controlled in vivo is through their rapid cell death in absence of the antigen. All mammals are exposed to a large array of persisting microbes, including their symbiotic relationship with gut flora. Such a mechanism could allow the compartmentalization of T reg cell function and prevent a general immunosuppression that would be associated with T reg cell dissemination.

The capacity of natural T reg cells to respond to parasitic antigen is further confirmed by the cell lines we have established from T reg cells from chronically infected mice. These cell lines maintain the characteristics of natural T reg cells in that they are $Foxp3^+$ and maintain suppressive function in vitro. Furthermore, the cell lines produce IL-10 and are able to expand 10–20 times per week when they encounter Leishmania-infected DCs. Self-antigen–specific cell lines have been successfully established in a model of murine diabetes, but for their expansion, they required high doses of IL-2 (2,000 U/ml) in the presence of anti-CD3 and anti-CD28 stimulation (26). It has been reported that T reg cells preferentially respond to DCs to proliferate in vitro and in vivo, but the in vitro expansion induced by DCs remained limited (27). In our model of infection, T reg cell lines specific for the parasite can be easily obtained when *L. major*–infected DCs were used as a source of APCs, suggesting that the parasite has specifically evolved to manipulate DCs in a manner that favors and sustains natural T reg cell proliferation. This is to our knowledge the first natural T reg cell line able to recognize foreign antigens. More importantly, these cell lines maintain their suppressive function in vivo. After transfer of T reg cell lines in chronically infected mice, mice undergo massive disease reactivation. We have previously shown that the transfer of natural T reg cells from chronically infected (and not naive) mice led to disease reactivation (10). In this report, the extent of such reactivation is dramatic because in less than 2 wk the parasite underwent massive expansion and dissemination to distal lymphoid organs. Notably, such parasite dissemination is also associated with massive

expansion of the natural T reg cell line that replaced the entire T cell compartment of the mice in all the lymphoid organs analyzed. Such expansion does not occur when the cell lines are transferred into naive mice or mice infected with *T. gondii*, further demonstrating the antigenic specificity of these cells. Thus, when natural T reg cells are activated and encounter their cognate antigen, these cells can powerfully out-compete other immune responses. These findings reinforce our previous hypothesis that in vivo, under natural conditions, natural T reg cells need to be eliminated rapidly in absence of antigen.

There is a growing body of literature demonstrating that natural T reg cells play a role in a large number of infections (7), including life-threatening diseases such as AIDS, hepatitis C virus, and malaria (18–20, 28–33). Strategies to manipulate natural T reg cell function or number have therapeutic potential. In a large number of infections in both mice and humans, the removal of natural T reg cells or molecules associated with their function has resulted in enhanced effector immune responses (10, 18, 20, 34). On the other hand, induction or activation of natural T reg cells represents a therapeutic objective when tissue damage is excessive (35, 36). Collectively, our results strongly support the idea that natural T reg cells can recognize microbial antigens and that this recognition is associated with their functions. Our results also demonstrate the devastating consequence of unleashed T reg cell function. In consequence, we believe that understanding the nature of the antigens recognized by natural T reg cells during infections is a necessary step in the design of rational strategies for controlling their function in vivo during infections.

MATERIALS AND METHODS

Mice. 6–8-wk old B/6 mice were purchased from Charles River. RAG2$^{-/-}$ B/6 and B6.SJL (Ly5.1, CD45.1) mice were obtained through the Taconic NIAID (NIH) Exchange Program. B/6 mice KO for MHC class II were purchased from The Jackson Laboratory. All mice were maintained at Children's Hospital Research Foundation Animal Facility or NIH animal house facility under pathogen-free conditions. All experiments on mice were performed in accordance with institutional guidelines (Cincinnati Children's Hospital Medical Center and NIAID).

Infection protocol and parasite quantitation. Infective-stage promastigotes (metacyclics) of *L. major* clone V1 (MHOM/IL/80/Friedlin) were isolated as described previously (37). Mice were infected in the ear dermis with 10^3 *L. major* metacyclic promastigotes using a 27 1/2 G needle in a volume of 10 μl. Parasite loads in the ears, spleens, and LNs were determined by limiting dilution of single-cell suspensions as described previously (38). Mice were infected with *T. gondii* as described previously (39). In brief, cysts of the avirulent ME49 strain of *T. gondii* were obtained from the brain of chronically infected B/6 mice. For experimental infection, 20 ME49 cysts in a volume of 0.5 ml were injected i.p.

In vitro restimulation assay of lymphocytes. LN or spleen single-cell suspensions from naive or Leishmania-infected mice were obtained as described above. CD4$^+$ T cells were preenriched by negative selection using magnetic beads (CD4$^+$ T cell isolation kit; Miltenyi Biotec). CD4$^+$CD25^{hi}, CD4$^+$CD25^{lo}, and CD4$^+$CD25$^-$ T cells were then purified by FACS as described previously (10). The T cell subsets were >98% pure as analyzed by flow cytometry. Isolated cells were labeled for 5 min at room temperature with 1.25 μM CFSE (Invitrogen) in PBS. 5×10^4 T cells were incubated with 2–5 $\times$ 10^5 BMDCs (40) in 200 μl RPMI containing 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, 55 μM 2-ME, and 10% FBS per well of a 96-well U-bottom plate. BMDCs were previously incubated overnight with or without *L. major* metacyclics (parasites/BMDCs ratio, 5:1) in the presence or absence of 50 ng/ml LPS from *Salmonella typhimurium* (Sigma-Aldrich) and 1 μg/ml anti-CD40 (clone 1C10; eBioscience). BMDCs were washed before culture with T cells. In some restimulation cultures, recombinant human TGF-β1 (Cell Sciences) was added at 2 ng/ml. After 3–5 d of T cell stimulation at 37°C in 5% CO_2, culture supernatants were collected for cytokine assays (see below) and the cells were analyzed by flow cytometry (see below).

Cytokine assays. IFN-γ, IL-2, IL-10, and GM-CSF were quantitated in culture supernatants of restimulated lymphocytes. The DuoSet ELISA system (R&D Systems) or alternatively, a multiplex assay (Linco Research), was used according to the manufacturer's protocol.

Flow cytometry analysis. Cells were fixed in 4% paraformaldehyde except when subsequent Foxp3 staining was performed. Fc receptors were blocked with 24G2 cell line culture supernatant, and the cells were stained with fluorochrome-conjugated anti-CD25 (clone PC61.5), anti-CD4 (clone RM4-5), and anti–TCR-β chain (clone H57-597) antibodies. In some experiments, cells were labeled for annexin V. The isotype controls used were rat IgG_1 (clone A110-1), rat IgG_{2a} (clone R35-95), and hamster IgG (clone Ha4/8), respectively. All antibodies were purchased from BD Biosciences. Incubations were performed for 20 min on ice. Cells were washed with PBS. For Foxp3 staining with the Foxp3 staining set (eBioscience), cells were treated according to the manufacturer's protocol. Cell acquisition was performed on a FACSCalibur flow cytometer using CELLQuest software (BD Biosciences). Cell proliferation was quantitated for the CD4$^+$TCR-β$^+$ population by analyzing the CFSE fluorescence pattern with ModFit LT software (Verity Software House).

BrdU and antibody treatments. Mice were treated daily for 4 d with 1 mg BrdU in 100 μl PBS i.p. Mice were killed the next day, and single-cell suspensions were prepared from the ears, LNs, and spleens as described above. Cells were fixed, permeabilized, digested with DNase, and stained with anti-BrdU antibodies (FITC BrdU flow kit; BD Biosciences) along with staining for CD4, TCR-β chain, and CD25 according to the manufacturer's protocol. Clinically cured mice were injected i.p. every 3 d with 1 mg monoclonal anti–IL-10 receptor antibody (DNAX; reference 41) or isotype control for a period of 3 wk.

Generation of Leishmania-specific T cell lines and adoptive cell transfers. CD4$^+$CD25$^+$ T cells were purified by cell sorting and seeded at 4×10^5 cells per well in 24-well plates in 1,200 μl RPMI containing 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM Hepes, 0.1 mM nonessential amino acid, 1 mM sodium pyruvate, 55 μM 2-ME, 10% FCS, and 10 ng/ml recombinant human IL-2 (T cell culture medium). T cells were stimulated, and weekly thereafter, with 2×10^5 Leishmania-infected BMDCs (except once every 4 wk to allow for T cell resting) in the presence of 4×10^6 erythrocyte-depleted spleen cells. Both infected BMDCs and spleen cells received an 8,000-rad γ-irradiation before culture with T cells. When T cell growth was microscopically visible, the cultures were diluted 1:2 in T cell culture medium until the end of the weekly expansion time. Expanded T cells were transferred i.v. into chronically infected mice or into mice inoculated at the same time in the ear with 10^3 *L. major* metacyclic promastigotes.

Statistical analysis. Statistical analysis was performed using Prism (GraphPad Software, Inc.). Dual comparisons were made using the unpaired Student's *t* test. All data from parasite numbers were log transformed before statistical tests were conducted.

Online supplemental material. Table S1 shows that CD4$^+$CD25^{hi} T cells from chronically infected mice are expanded when exposed to *L. major*–infected DCs.

Fig. S1 shows the coexpression of Foxp3 and activation markers on lymphocytes purified from chronically infected mice. Table S1 and Fig. S1 are available at http://www.jem.org/cgi/content/full/jem.20052056/DC1.

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