TCR ζ Down-Regulation under Chronic Inflammation Is Mediated by Myeloid Suppressor Cells Differentially Distributed between Various Lymphatic Organs[1]

Analía V. Ezernitchi,* Ilan Vaknin,* Leonor Cohen-Daniel,* Ofer Levy,* Efrat Manaster,* Amal Halabi,[†] Eli Pikarsky,[‡] Lior Shapira,[†] and Michal Baniyash[2]*

T cell AgR ζ chain down-regulation associated with T cell dysfunction has been described in cancer, infectious, and autoimmune diseases. We have previously shown that chronic inflammation is mandatory for the induction of an immunosuppressive environment leading to this phenomenon. To identify the key immunosuppressive components, we used an in vivo mouse model exhibiting chronic inflammation-induced immunosuppression. Herein, we demonstrate that: 1) under chronic inflammation secondary lymphatic organs display various immunological milieus; ζ chain down-regulation and T cell dysfunction are induced in the spleen, peripheral blood, and bone marrow, but not in lymph nodes, correlating with elevated levels of $Gr1^{+}Mac\text{-}1^{+}$ myeloid suppressor cells (MSC); 2) MSC are responsible for the induction of such an immunosuppression under both normal and inflammatory conditions; and 3) normal T cells administered into mice exhibiting an immunosuppressive environment down-regulate their ζ expression. Such an environment is anticipated to limit the success of immunotherapeutic strategies based on vaccination and T cell transfer, which are currently under investigation for immunotherapy of cancer. ***The Journal of Immunology,* 2006, 177: 4763–4772.**

Down-regulation of TCR ζ chain expression associated with T cell dysfunction was described in various pathologies, such as advanced cancer (1–9), chronic infections (10, 11), and autoimmune diseases (12–14). The fact that pathologies that differ in their physiology and etiology show decreased ζ chain expression and exhibit T cell dysfunction suggests the existence of a common denominator linking these conditions. In our previous studies, we identified sustained exposure to Ag and chronic inflammation, which are characteristics shared by the different pathologies, as being the key factors responsible for the induction of ζ chain down-regulation and impaired T cell function (15). This was shown by means of an in vivo model system, in which normal mice were repeatedly exposed to heat-killed bacteria that induce chronic inflammation, leading to ζ chain down-regulation and impaired T cell function. The generated immunosuppressive conditions affected all T cells within the spleen with no relation to their antigenic specificity. Under these conditions, ζ chain is the sole TCR subunit to be affected, as determined in T cells isolated from hosts with the above-mentioned pathologies (15). Moreover, we demonstrated that ζ chain expression under the generated immunosuppressive conditions is regulated at the post-translational level, targeted primarily to lysosomal degradation (15). Thus, our cumulative data suggest that the experimental system we developed could serve as a general model for chronic inflammation-induced immunosuppression, mimicking the inflammatory conditions generated in hosts bearing various tumors, infections, and autoimmune diseases. Moreover, our preliminary studies revealed that non-T cells isolated from spleens of the treated mice have the capacity to induce ζ down-regulation when coincubated in vitro with normal T cells (15). This suggests that an immunosuppressive environment is created in hosts that develop a chronic inflammatory immune response. However, the nature of the cells that induce this phenomenon, as well as their in vivo localization and mode of action, are not known. In the present study, we used our model system (15) to explore the nature of the immunosuppressive cells operating under chronic inflammatory conditions and to define their in vivo localization and characteristics.

We demonstrate that the consequences of chronic inflammation differ in various secondary immune organs. In the spleen, peripheral blood and bone marrow, an immunosuppressive environment is generated inducing down-regulation of ζ chain expression and impaired T cell function. However, within the lymph nodes the TCR ζ expression levels and immune functions remain normal. In addition, we provide evidence that the immunosuppressive cell population responsible for the induction of ζ down-regulation and impaired T cell function is composed of $Gr1^{+}Mac\text{-}1^{+}$ myeloid suppressor cells (MSC).[3] While this cell population accumulates in the spleen, peripheral blood, and bone marrow of the affected mice, their amounts are minute in the lymph nodes of the same mice. The results also show that the induction of TCR ζ chain down-regulation depends upon MSC to T cell ratios and the MSC's activation state. We provide evidence that the generated

*The Lautenberg Center for General and Tumor Immunology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel; [†]Department of Periodontology, The Hebrew University-Hadassah Faculty of Dental Medicine, Jerusalem, Israel; and [‡]Department of Pathology, Hadassah-Hebrew University Medical Center, Jerusalem, Israel

Received for publication February 23, 2006. Accepted for publication July 12, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported by the Israel Academy of Sciences and Humanities, by the Israeli Ministry of Health, by the Israel Cancer Association, by the Joint German-Israeli Research Program (Deutsches Krebsforschungszentrum), Israel Cancer Research Fund, and by the Herman Elbin and The Joseph and Matilda Melnick funds.

[2] Address correspondence and reprint requests to Dr. Michal Baniyash, The Lautenberg Center for General and Tumor Immunology, The Hebrew University-Hadassah Medical School, P.O. Box 12272, Jerusalem 91120, Israel. E-mail address: baniyash@cc.huji.ac.il

[3] Abbreviation used in this paper: MSC, myeloid suppressor cell.

Copyright © 2006 by The American Association of Immunologists, Inc.
0022-1767/06/$02.00

immunosuppressive environment negatively affects newly injected normal T cells that reach the spleen, inducing ζ down-regulation within 24 h upon their administration. In contrast, cells reaching the lymph nodes were not affected. The clinical implications and the relevance of the immunosuppressive environment to the immunotherapeutic regimens that are in practice today are discussed.

Materials and Methods

Animals

Female BALB/C mice, 6–7 wk of age were bred at the Hebrew University pathogen-free facility. Animal use followed protocols approved by the Hebrew University-Hadassah Medical School Institutional Animal Care and Use Committee.

The in vivo chamber model system

Our in vivo experimental mouse model system was described previously (Ref. 15; see Fig. 1*A*). Briefly, chambers constructed from coils of titanium wire (16, 17) were implanted subcutaneously in normal (healthy) mice. After 1 wk mice were s.c. injected with 0.1 ml of 1×10^{10} CFU/ml of heat-killed Gram-negative bacteria (*Porphyromonas gingivalis*) (18–20) mixed with 0.1 ml of IFA (Sigma-Aldrich). After 1 and 2 wk, the mice received an intrachamber injection of heat-killed *P. gingivalis* (0.1 ml of 1×10^{10} CFU/ml) in PBS. Unless stated otherwise, peripheral blood, spleens, lymph nodes, and bone marrow were collected 1 day after the last injection (day +1). Control mice were subjected to the same protocol, but this group of mice was injected with PBS instead of *P. gingivalis*.

Cell labeling and injection

Splenocytes isolated from normal mice were resuspended in diluent C (Sigma-Aldrich) at a concentration of 2×10^8/ml. An equal volume of the fluorescent dye PKH-26 (Sigma-Aldrich) prepared in diluent C was added to the cell suspension, mixed well by gentle inversion of the tube, and incubated at room temperature for 3 min with continuous agitation. Cells were protected from the light during the entire procedure. The staining reaction was stopped by the addition of an equal volume of 1% BSA in Hanks (without phenol red). The cells were centrifuged at 400 × *g* for 10 min at 25°C, the supernatant was discarded, and the cell pellet was washed in Hanks, counted, and resupended to the desired concentration. A control sample was prepared in diluent C only. A sample of the cells was taken for FACS analysis. The labeled cells, designated as donor cells, were resuspended in HBSS and 30×10^6 cells in 100 μl of buffer were injected intrasplenically or i.v. into acceptor (host) mice that were either *P. gingivalis* treated or control. The procedure was performed under sterile conditions.

Flow cytometry analysis

For cell surface labeling, cell samples were washed and resuspended in 100 μl of FACS buffer (1% BSA in PBS) containing FcR blocking Abs (24G2) and specific labeled Abs directed against: Thy1.2, CD3 ε, Gr1, and Mac-1 (BD Pharmingen) or TCR ζ (H146 mAb provided by Dr. R. Kubo, La Jolla Institute for Allergy and Immunology, La Jolla, CA). The cells were then washed with FACS buffer, and specific samples were labeled with a second reagent (streptavidin conjugated to PE or to Cy5) and washed. Samples were analyzed by flow cytometry on a BD Biosciences FACSCalibur. For intracellular Ag staining, cells were fixed with 1% paraformaldehyde for 20′ at 4°C washed and permeabilized with 200 μl of 0.1% saponin in PBS for 30 min at 4°C in dark. Incubations with Abs were then performed as described above.

Proliferation test

Cells isolated from the spleen and lymph nodes were cultured (2×10^6 cells/ml) during 48 h in the presence of 1 μg/ml CD3ε Abs, 2.5 μg/ml Con A, or 2 ng/ml PMA combined with 0.2 μM calcium ionophore. During the last 3 h of activation, 15 μM BrdU (Sigma-Aldrich) was added. Cells were collected, precoated with anti-mouse CD16/CD32, and stained with biotinylated anti-Thy1.2, followed by incubation with Cy5-conjugated streptavidin. Cells were then washed, diluted in cold 0.15 M NaCl, and fixed with cold 95% ethanol. After 30 min on ice, cells were washed with PBS, incubated for 30 min at room temperature in PBS containing 1% paraformaldehyde and 0.01% Tween 20. Cells were centrifuged, incubated for 10 min at room temperature with DNase I solution, washed, and then incubated for 30 min with FITC-conjugated anti-BrdU. Following washing, washed the cells were subjected to flow cytometry analysis. Nonpulsed activated cells were used as a control for the anti-BrdU staining.

Paraffin tissue section staining

Five-micrometer, formalin-fixed and paraffin-embedded sections from the spleens and lymph nodes were deparaffinized and rehydrated. After endogenous peroxidase quenching (H_2O_2), Ags were retrieved by boiling the sections in 10 mM citrate buffer (pH 6.0). Immunostaining was then performed using the specific anti-Gr1 and anti-ε Abs, and the appropriate second reagents were used (Zymed Laboratories) to visualize the labeled cells.

Cell isolation and separation

Mice were sacrificed, spleen, bone marrow, and lymph nodes were excised, and cell suspension was obtained (15). For magnetic column separation, cells were first labeled (30 min at 4°C) with a specific Ab conjugated to FITC or biotin. The cells were then washed with buffer (PBS with 0.5% BSA, 0.4% EDTA, labeled (45 min at 4°C) with anti-FITC or anti-biotin Abs conjugated to magnetic microbeads (Miltenyi Biotec), washed, and loaded onto a column placed in the magnetic field. Negatively and positively selected cell populations were isolated according to the manufacturer's directions (Miltenyi Biotec). For separation according to the cell density, Percoll gradient was used. Percoll (Amersham Biosciences) was diluted in PBS to form a step gradient of 70, 60, 50, and 40% in a conical tube, and the cells were layered on the bottom. The tube was centrifuged for 30 min at 1800 × *g* at 4°C. Cells were then collected from each fraction, washed twice, and analyzed.

Ex vivo coincubation experiments

T cells isolated from spleen were suspended in complete RPMI 1640 medium and coincubated at 37°C, with the $Gr1^+Mac\text{-}1^+$-enriched cell fraction obtained from the spleen or bone marrow of *P. gingivalis*-treated, control, or normal mice. After 24 h of incubation, cells were harvested and the level of TCR ζ chain expression was detected by flow cytometric analysis. Transwell inserts were used with 0.02-μm diameter pores (Nunc), when indicated. Proliferation test applied to the coincubation experiments was performed by adding the specific activators together with the cell counterparts at the very start point of the incubation.

Results

Differential TCR ζ chain expression and T cell function in spleen and lymph nodes of mice subjected to chronic inflammatory conditions

To evaluate the effect of a chronic inflammatory immune response on T cells in different secondary lymphatic organs, we used our previously described in vivo experimental system (15). Normal mice were repetitively injected with heat-killed *P. gingivalis* bacteria (Fig. 1*A*), and 1 day after the last injection, T cells from the spleen, peripheral blood, and lymph nodes were analyzed for TCR ζ chain expression and T cell function. As shown in Fig. 1*B*, under these conditions, mice developed splenomegaly, reaching at least twice the size of spleens from control mice, and splenic T cells from the treated mice showed reduced levels of ζ chain expression (Fig. 1*C*). ζ expression levels were also diminished in T cells derived from peripheral blood, although to a lesser degree than in splenic T cells (Fig. 1*C*). Surprisingly, in contrast to the impaired ζ expression in the splenic and peripheral blood T cells, ζ expression in T cells localized within the lymph nodes was not affected (Fig. 1*C*), even though the nodes were significantly enlarged (Fig. 1*B*). In all T cell populations tested, the sole TCR subunit to be affected was the TCR ζ chain. CD3 ε expression (Fig. 1*C*, *lower panels*) and expression of the other TCR subunits (data not shown) was not down-regulated. Moreover, we found a direct correlation between ζ chain expression and in vitro T cell function in the different lymphatic organs. While splenic T cells derived from treated mice showed a diminished proliferative response to stimulation with anti-CD3 Abs or Con A in comparison with control splenic T cells, lymphocytes in the lymph nodes of the same treated mice were functionally normal (Fig. 1*D*). In these analyses, the proliferative response was assessed by evaluating T cell-specific BrdU incorporation using flow cytometry analysis. This enabled us to focus specifically on T cell responses and to ignore

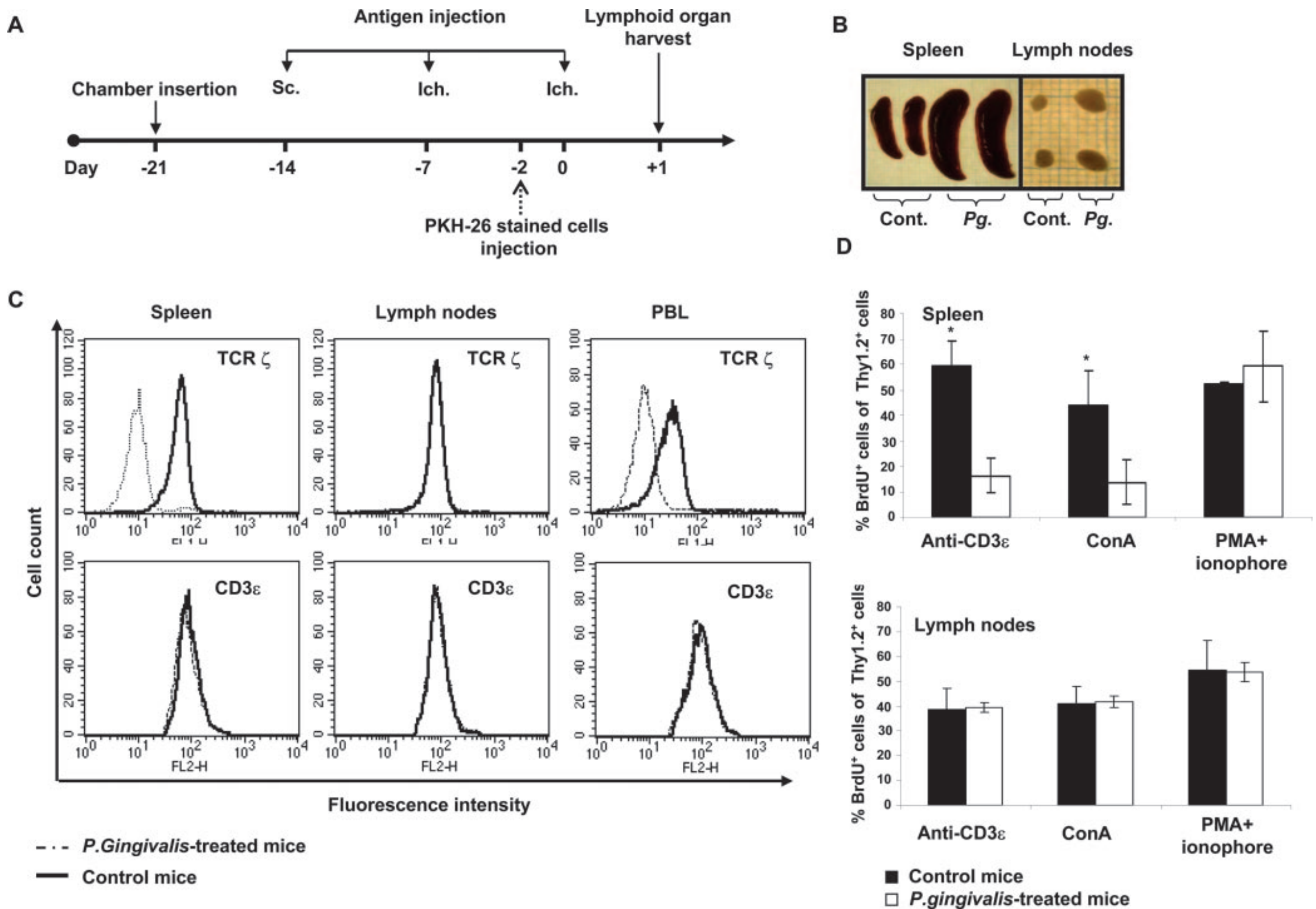

FIGURE 1. T cells within the spleens, peripheral blood, and lymph nodes of *P. ginigivalis*-treated mice are differentially suppressed. *A*, The mouse model system for chronic inflammation. On day −21, a titanium chamber is inserted into normal mice. On day −14, mice are injected subcutaneously (Sc.) with *P. gingivalis* in IFA. On day −7 and 0, heat-killed *P. gingivalis* in PBS is injected into the chamber (Ich.). One day after the last Ag injection (day +1), peripheral blood, spleens and lymph nodes are harvested, cells are isolated and analyzed. When indicated (day −2), PKH-labeled splenocytes were administrated. *B*, Spleen and lymph nodes from *P. gingivalis*-treated (*Pg.*) or control (Cont.) mice were isolated one day after the last Ag injection and photographed. *C*, Cells were isolated from the spleen, lymph nodes, and peripheral blood of control and *P. gingivalis*-treated mice. The cells were fixed, permeabilized, and double labeled with anti-Thy1.2 and anti-ζ or anti-CD3ε Abs and analyzed by flow cytometry. The histograms show ζ and CD3ε expression in Thy1.2$^+$ T cells. *D*, Splenocytes and lymph nodes from control and *P. gingivalis*-treated mice were activated with anti-CD3 Abs, Con A (conA), or a combination of PMA and calcium ionophore. Specific T cell proliferation was evaluated by flow cytometry, measuring BrdU incorporation in Thy1.2$^+$ cells. The results are presented as the mean value of three independent experiments, and SDs are shown. Cells from the spleen of *P. gingivalis*-treated mice show a significantly lower response to stimulation with anti-CD3 and Con A compared with the cells from control mice; *, $p < 0.002$, according to Student's *t* test.

other proliferating cell populations that could affect the results as in cases where thymidine incorporation is used. As shown in Fig. 1*D*, down-regulated ζ expression was correlated with an impaired T cell response to activators such as anti-CD3 Abs and Con A that require an intact and functional TCR. Although T cells from the spleen of *P. gingivalis*-treated mice could produce normal levels of the cytokines IL-2 and IFN-γ upon activation via the TCR, they express lower levels of IL-2R when compared with T cells from the nontreated mice (data not shown), and their proliferative response is impaired (Fig. 1*D*). In contrast, activation with PMA + calcium ionophore, which bypasses the TCR, lead to a normal proliferative response in all tested cells. This indicates that the signal transduction machinery downstream of the TCR in T cells from the spleen of the treated mice is normal (Fig. 1*D*). Thus, under chronic inflammation, T cells within different lymphatic organs are differently affected; T cells in the spleen down-regulate TCR ζ expression and their in vitro function is impaired, and in the lymph nodes of the same mice, T cells exhibit normal features of TCR ζ expression and function.

Mice under chronic inflammatory conditions exhibit disorganized architecture of the spleen

Since T cells in the spleen and lymph nodes of *P. gingivalis*-treated mice were differently affected, we next examined the morphology and structure of these organs. As described above, the treatment with *P. gingivalis* induced splenomegaly and enlargement of lymph nodes (Fig. 1*B*). The spleen and lymph nodes are organized in compartmentalized follicular structures, which optimize cellular interactions. Thus, an abnormal organization is expected to affect T cell function (21). The histological analysis revealed that spleens from *P. gingivalis*-treated mice (Fig. 2*A*, I) exhibited disrupted architecture of the spleen when compared with spleens of control mice (Fig. 2*A*, II). The spleens isolated from *P. gingivalis*-treated mice displayed expansion of the red pulp by sheets of white blood cells, while the white pulp was not altered compared with controls. This disorganized appearance was restored to normal within 10 days after the last Ag injection, correlating with the recovery of ζ chain expression (data not shown). In contrast, lymph nodes

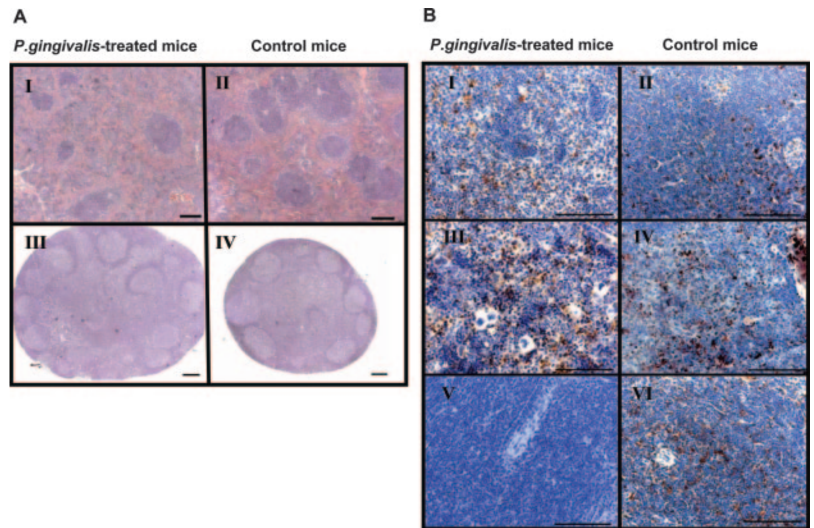

FIGURE 2. Structure of spleen and lymph nodes of *P. gingivalis*-treated and control mice. Paraffin-embedded sections from spleen (*A*, I and II; *B*, III–VI) and lymph nodes (*A*, III and IV; *B*, I and II) from *P. gingivalis*-treated and control mice were stained with hematoxylin (*A*) and with anti-CD3ε Abs for the localization of T cells in the lymph nodes (*B*, I and II) and the red (*B*, III and IV) and white (*B*, V and VI) pulp zones of the spleen, as described in *Materials and Methods*. Magnification (in *A*) ×100, (in *B*) ×400.

isolated from the treated mice, although enlarged, as expected in continuously immunized mice, showed follicular hyperplasia in both treated and control groups (Fig. 2*A*, III and IV). The spleen is separated into two major compartments, the red and white pulp zones. The red pulp is mainly populated by macrophages and is the zone in which erythrocytes are eliminated. The white pulp, on the other hand, contains mainly lymphocytes and dendritic cells, which are separated from the red pulp by the marginal sinus (22). We therefore examined the distribution of T cells in the spleens and lymph nodes using immunohistochemical analysis with anti-CD3ε Abs (Fig. 2*B*). There was no difference in the T cell abundance or distribution between the lymph nodes of *P. gingivalis*-treated (Fig. 2*B*, I) and control (Fig. 2*B*, II) mice. Remarkably, analysis of spleens from *P. gingivalis*-treated mice showed that T cells were excluded from the white pulp (Fig. 2*B*, V) but were abundant in the expanded red pulp (Fig. 2*B*, III). This is in contrast with control mice in which T cells are present in both the red pulp (Fig. 2*B*, IV) and white pulp (Fig. 2*B*, VI). Taken together, the splenomegaly, reduced ζ expression, impaired T cell function, and the unusual localization of T cells within the spleen of the treated mice, all appear to be associated with the impaired immune status observed under chronic inflammatory immune conditions. This raises the hypothesis that the impaired T cell function is brought about by cell-cell interactions, which take place in the expanded red pulp of the *P. gingivalis*-treated spleens.

Differential distribution of myeloid cells between the spleen and lymph nodes of mice during chronic inflammation

Since T cells are differently affected in the spleen and lymph nodes, we next examined possible differences in the presence of various cell populations between the spleen and lymph nodes in the treated vs the control mice. Our results revealed that in the spleen, while the ratio between T and B cells remained similar in the treated and control mice, a dramatic augmentation in the number of non-T non-B cells appeared in *P. gingivalis*-treated mice (Fig. 3*A*, *upper panel*). In contrast, within the lymph nodes, the levels of the non-T non-B cell populations remained small in both treated and non-treated mice (Fig. 3*A*, *lower panel*). The only change observed in the lymph nodes of the treated mice was an increase in the B cell number due to the strong immune response, thus affecting the ratios between T and B cells. Analysis of the splenic non-T non-B cell population revealed that it is primarily composed of $Gr1^+Mac\text{-}1^+$ double-positive cells that correspond to 38% of the splenocytes in the treated mice (Fig. 3*B*, *upper panel*). The proportion of $Gr1^+Mac\text{-}1^+$ cells in the spleen of control mice was much smaller (6% of the splenocytes) (Fig. 3*B*, *upper panel*),

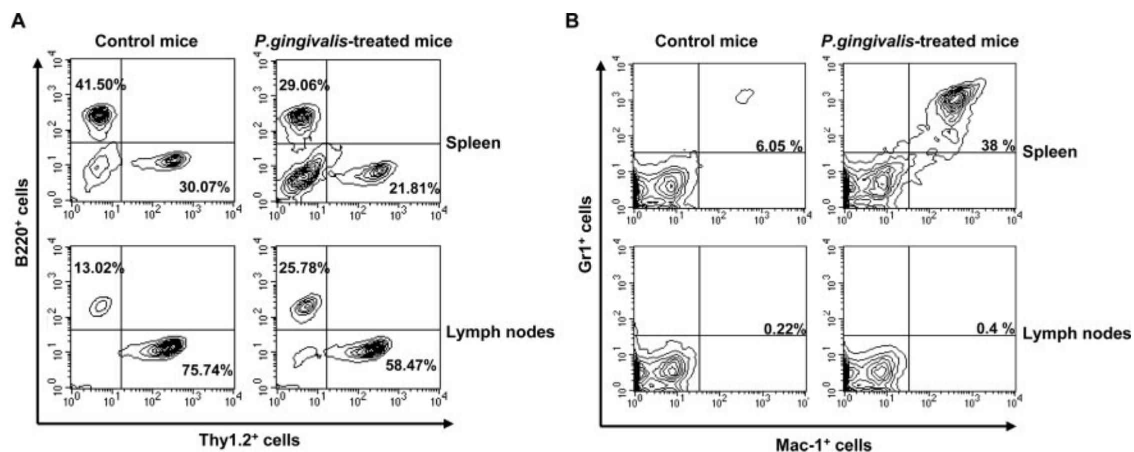

FIGURE 3. Spleen and lymph nodes of *P. gingivalis*-treated mice differ in their immune cell populations. Spleen and lymph nodes from *P. gingivalis*-treated and control mice were harvested, and a cell suspension was prepared and double stained with specific Abs for the detection of $B220^+$ B cells and $Thy1.2^+$ T cells (*A*) and the detection of Gr-1 and Mac-1 double-positive cells (*B*). All samples were subjected to flow cytometry analysis. The results presented are of a representative experiment out of five performed.

and these cells were not found in the lymph nodes of either control or treated mice (Fig. 3B, *lower panels*). An elevated percentage of the Gr1$^+$Mac-1$^+$ cells was observed as well in the peripheral blood of the treated mice when compared with control mice (~50–60%, data not shown). Previous studies characterized the Gr1$^+$Mac-1$^+$ cells as a mixture of mature and immature MSC (23, 24). Thus, the differences in ζ expression and T cell function between the peripheral blood, spleen, and lymph nodes of *P. gingivalis*-treated mice could be explained by the increased percentage of the Gr1$^+$Mac-1$^+$ cells in the spleens of the treated mice.

Gr1$^+$Mac-1$^+$ cells are responsible for ζ down-regulation and impaired T cell function observed in mice with chronic inflammation

To assess whether the Gr1$^+$Mac-1$^+$ cells derived from the spleen of *P. gingivalis*-treated mice affect ζ expression levels, we performed ex vivo coincubation experiments. T cells isolated from normal mice were exposed in vitro to the Gr1$^+$Mac-1$^+$-enriched cell population derived from the spleen of *P. gingivalis*-treated and control mice at a ratio of 1:2. This ratio represents the distribution of the two cell populations (T cells ~13–22% and Gr1$^+$Mac-1$^+$ cells ~35–45%) in the spleens of the treated mice. Following 24 h of coincubation with the Gr1$^+$Mac-1$^+$ enriched cell population isolated from the spleen of the treated mice, normal T cells dramatically down-regulated their TCR ζ expression (Fig. 4*A*). Surprisingly, T cells coincubated with the Gr1$^+$Mac-1$^+$ cells isolated from the spleen of control mice at a ratio of 1:2, also down-regulated TCR ζ expression, although to a lesser extent than that observed using the Gr1$^+$Mac-1$^+$ cells from the spleen of the treated mice (Fig. 4*A*). While Gr1$^+$Mac-1$^+$ cells within the spleen of the control mice did not affect ζ chain expression levels of the endogenous T cells (Fig. 1*C*), they were able to induce ζ down-regulation in the in vitro coincubation experiments (Fig. 4*A*). Based on our results, the difference between the in vivo and the in vitro effect of Gr1$^+$Mac-1$^+$ cells from the control mice is most likely due to the differences in the ratio between the T and Gr1$^+$Mac-1$^+$ cells. While in the spleen of control mice the ratio between the T and Gr1$^+$Mac-1$^+$ cells is 5:1 (Fig. 3*B*), in the coincubation experiments the ratio is 1:2, similar to the ratio observed in the spleen of the treated mice. To confirm that the Gr1$^+$Mac-1$^+$ cells represent the primary cell population that induces ζ down-regulation, we performed depletion experiments. To this end, splenocytes isolated from *P. gingivalis*-treated mice were initially depleted of T cells and then separated from Gr1$^+$Mac-1$^+$ cells. The Gr1$^+$Mac-1$^+$ and Gr1$^-$Mac-1$^-$ cells were then coincubated with normal T cells. As shown in Fig. 4*B*, splenocytes depleted of Gr1$^+$Mac-1$^+$ cells did not affect ζ expression, while the Gr1$^+$Mac-1$^+$ cells strongly induced down-reglation of ζ expression in T cells. These results indicate that the Gr1$^+$Mac-1$^+$ cells are responsible for the induction of ζ down-regulation. Moreover, the Gr1$^+$Mac-1$^+$ cells isolated from the spleen of the treated mice induced a more pronounced ζ down-regulation than those isolated from control mice (Fig. 4*A*), indicating that activation by *P. gingivalis* immunization enhances their immunosuppressive activity. The effect of the MSC on TCR ζ expression is cell contact dependent or requires a close proximity between the cell counterparts as shown in Fig. 4*C*, where MSC and T cells were coincubated in the presence of a Transwell insert, which impedes the contact between these two cell populations. The immunosuppressive effect of the MSC on T cells is reflected by ζ down-regulation, correlating with unresponsiveness to TCR-mediated T cell activation, as indicated by BrdU incorporation (Fig. 4*D*).

Since Gr1$^+$Mac-1$^+$ cells originate in the bone marrow and their concentration there is significantly elevated, our next step was to analyze the effect of the Gr1$^+$Mac-1$^+$-enriched cell population isolated from the bone marrow of both treated or control mice on ζ expression of normal T cells. Gr1$^+$Mac-1$^+$ cells isolated from the bone marrow exhibited a similar immunosuppressive activity to that of spleen-derived cells. However, the Gr1$^+$Mac-1$^+$ cells isolated from the bone marrow of treated mice induced a more pronounced ζ down-regulation than that induced by the cells isolated from control mice (Fig. 5*A*). Moreover, we demonstrate that endogenous T cells isolated from the bone marrow of normal mice, in which the ratio between T and Gr1$^+$Mac-1$^+$ cells is 1:16, express significantly lower levels of ζ chain than T cells isolated from the spleen of the same mice in which the ratios of T to

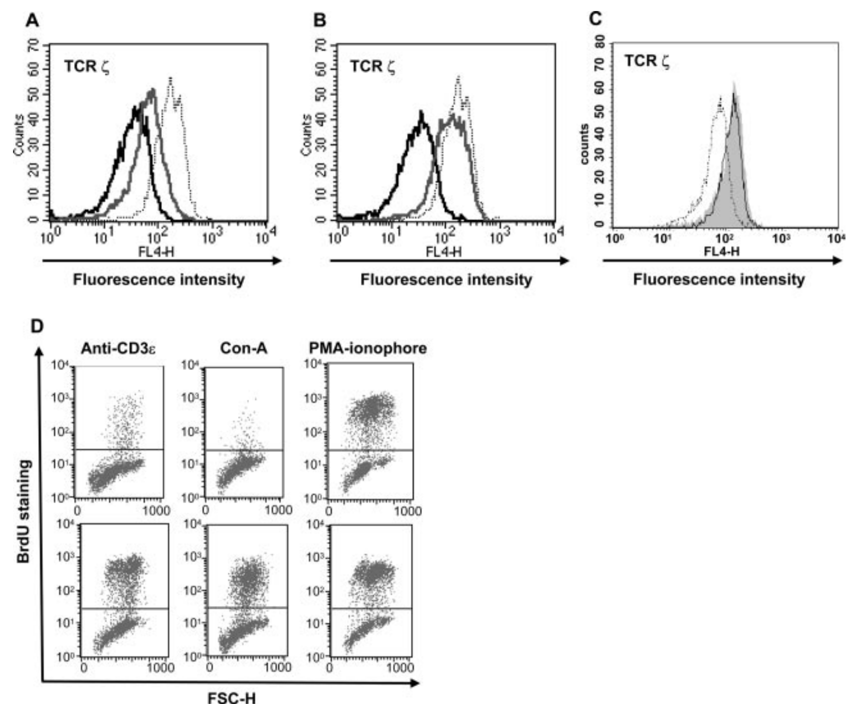

FIGURE 4. Splenic Gr1$^+$Mac-1$^+$ double-positive cells induce ζ chain down-regulation in normal T cells. *A*, Normal splenic T cells were isolated and coincubated with splenic Gr1$^+$Mac-1$^+$-enriched cells (as described in *Materials and Methods*) from control (continuous gray line) and *P. gingivalis*-treated mice (continuous black line) at a ratio of 1:2. Following 24 h, T cells were stained for the detection of ζ chain expression (continuous lines) and compared with ζ expression in the T cells before the coincubation (dashed line). *B*, Normal T cells (dashed line) were coincubated with splenic Gr1$^+$ cells (continuous black line) and with splenocytes depleted of Gr1$^+$ cells (continuous gray line) derived from *P. gingivalis*-treated mice at a ratio of 1:2. *C*, Normal splenic T cells were coincubated with normal splenic non-T cells (gray background) or with Gr1$^+$Mac-1$^+$-enriched cells derived from *P. gingivalis*-treated mice in the presence (continuous line) or absence of Transwell inserts (dashed line). *D*, Normal T cells were coincubated with splenic Gr1$^+$Mac-1$^+$-enriched cells derived from *P. gingivalis*-treated mice (*upper panels*) or normal spleen cells (*lower panels*) together with the different activators. Proliferation of T cells was measured by BrdU incorporation as described in Fig. 1*D*.

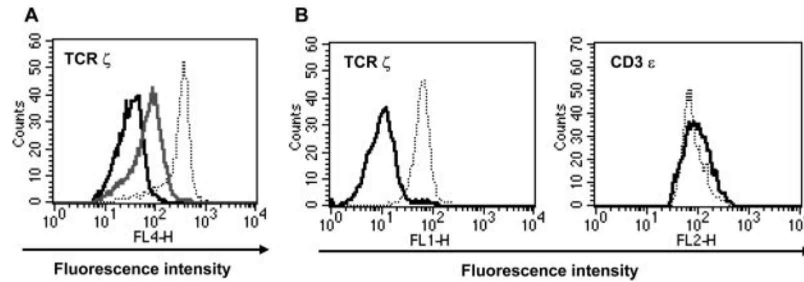

FIGURE 5. Gr1$^+$ Mac-1$^+$ double-positive cells from the bone marrow of *P. gingivalis*-treated, control, and normal mice induce ζ chain down-regulation in normal T cells. *A*, Normal splenic T cells were isolated and coincubated with Gr1$^+$Mac-1$^+$-enriched cells from the bone marrow of control (continuous gray line) and *P. gingivalis*-treated mice (continuous black line) at a ratio of 1:2. Following 24 h, T cells were stained for the detection of ζ chain expression and compared with ζ expression in the T cells before the coincubation (dashed line). *B*, T cells isolated from the bone marrow (continuous line) and the spleen of normal mice (dashed line) were analyzed for the expression of ζ (*left panel*) and CD3ε (*right panel*) chains by using specific anti-ζ and anti-ε Abs and flow cytometry analysis.

Gr1$^+$Mac-1$^+$ cells is 10:1 (Fig. 5*B*). It is important to note that in contrast to the affected expression of the ζ chain, the CD3ε expression in the T cells localized within the bone marrow of normal mice was not affected (Fig. 5*B*). These results indicate that elevated levels of normal Gr1$^+$Mac-1$^+$ cells are able to induce a suppressive environment that impairs TCR ζ chain expression when mixed with T cells. Furthermore, these cells are more potent under chronic inflammatory conditions due to their enhanced activation state. Thus, our results suggest that the Gr1$^+$Mac-1$^+$ cells generate the in vivo immunosuppressive environment observed in the treated mice.

The immunosuppressive environment generated in the spleen of P. gingivalis-*treated mice induces TCR ζ chain down-regulation of normal administered T cells*

The knowledge that an immunosuppressive environment is generated in the course of developing chronic inflammatory responses has major significance for pathologies in which immunotherapies, such as T cell therapy and/or vaccination protocols, are applied. The immunosuppressive environment that negatively affects the endogenous immune system is also expected to damage newly administered T cells. To test this hypothesis, we took advantage of our results showing that the lymph node and spleen environments of the treated mice differentially affect T cell ζ chain expression levels. To examine how normal T cells are affected when exposed in vivo to both the spleen and lymph node environments, we performed cell transfer experiments, mimicking T cell-mediated therapy. Based on the kinetics of the *P. gingivalis* experimental system, normal mouse donor cells labeled with the PKH-26 fluorescent dye were transferred by intrasplenic injection to treated or control host mice 2 days before final Ag injection (day −2; Fig. 1*A*). ζ expression levels in donor T cells localized within the spleen and lymph nodes of the *P. gingivalis*-treated mice were compared with the donor cells localized in control mice, the latter serving as 100% expression at each time point (Fig. 6, *A* and *B*). In parallel, ζ expression levels were analyzed in the spleen and lymph nodes of the host mice (*P. gingivalis*-treated and control), ζ expression in the control mice serving as 100% at each time point (Fig. 6, *C* and

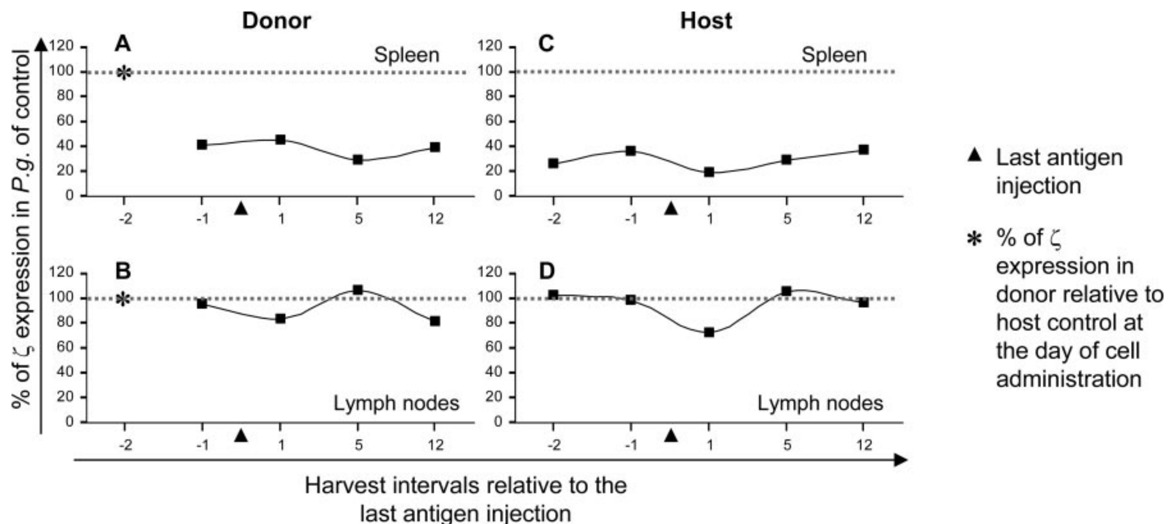

FIGURE 6. The effect of chronic inflammation on ζ expression in adoptively transferred normal T cells. PKH-26 labeled splenocytes derived from control mice (donor cells) were injected into the spleen of *P. gingivalis*-treated or control mice (hosts). After 24 h and at the indicated time points, spleens and lymph nodes of the host were harvested and cell suspension was double stained for Thy1.2 and ζ expression. The ζ expression was detected in T cells that were found within the PKH-26$^+$ (donor *A* and *B*) or PKH-26$^-$ population (host *C* and *D*). ζ expression of donor cells localized within the control mice (*A* and *B*) and of the hosts' control mice (*C* and *D*) were designed as 100% (discontinuous gray lines). Accordingly, ζ expression levels of the donor cells localized within the *P. gingivalis*-treated mice and of the *P. gingivalis*-treated host cells are presented as percentage of ζ expression in *P. gingivalis* of control and calculated as ((median of ζ expression in *P.g.* mice/median of ζ expression of control mice) × 100) for each day (■) in spleen (*A* and *C*) and lymph nodes (*B* and *D*). (*) asterisks represent the percentage of ζ expression of the donor cells relative to the control at the time of administration. In this figure, cells from control mice expressing 100% of ζ were injected into the different hosts.

D). We demonstrate that the normal donor cells localized in the spleen of *P. gingivalis*-treated mice decreased their ζ expression levels within 1 day after exposure to the host's splenic environment (Fig. 6*A*). In contrast, the donor cells localized within the lymph nodes of *P. gingivalis*-treated mice were not affected and their ζ chain expression levels remained normal (Fig. 6*B*). The normal donor cells localized in both the spleen and lymph nodes of control mice expressed normal ζ levels designated as 100% (Fig. 6, *A* and *B*, discontinuous line). Thus, ζ expression levels of the donor T cells correlate with those of the hosts; ζ expression levels in the *P. gingivalis*-treated mice remained low in the spleen during the different time points when compared with the control mice (Fig. 6*C*), while ζ expression levels in the lymph nodes of both treated and control mice remained normal (Fig. 6*D*). Similar in vivo experiments were performed using i.v. instead of intrasplenic administration, and comparable results were obtained (data not shown).

Affected splenic T cells exit the spleen, migrate to the lymph nodes and regain their ζ chain expression

We next assessed whether T cells, which lost their ζ chain upon exposure to the splenic environment of *P. gingivalis*-treated mice, are able to exit the spleen and migrate to the lymph nodes. Although in previous experiments (Fig. 6) we visualized normal donor T cells within the lymph nodes following intrasplenic injection to *P. gingivalis*-treated mice, these cells could represent a subpopulation of T cells that never reduced their levels of ζ chain upon the entry into the spleen of treated mice. To address this issue, we performed cell transfer experiments in which splenocytes isolated from *P. gingivalis*-treated mice were PKH labeled, and served as the donor cells. The cells were injected into the spleen of *P. gingivalis*-treated or control mice (Fig. 7). As described for Fig. 6, ζ expression levels in donor T cells that are localized within the spleen and lymph nodes of *P. gingivalis*-treated mice were compared with those localized in control mice, the latter serving as 100% ζ expression at each time point (Fig. 7, *A* and *B*). In parallel, ζ expression levels were analyzed in the spleen and lymph nodes of the host mice (*P. gingivalis*-treated and control), when ζ expression in the control mice served as 100% (Fig. 6, *C* and *D*). Our results indicate that donor cells, characterized by a low level of ζ chain expression at the time of the intrasplenic injection, when remaining in the spleen of *P. gingivalis*-treated mice they retain their down-regulated ζ expression levels (Fig. 7*A*). In contrast, we demonstrate that T cells with down-regulated ζ expression levels are able to exit the spleen, reach the lymph nodes, and regain their ζ expression (Fig. 7*B*). As can be seen in Fig. 7, *C* and *D*, ζ expression levels in the donor cells reflect those obtained in the hosts' spleens and lymph nodes. Thus, affected T cells characterized by reduced ζ chain expression do not lose their capacity to migrate between the lymphatic organs. These experiments provide evidence of the recovery of T cells in the lymph nodes. As described above, analysis of T cells derived from PBL reveled that the cells exhibit reduced levels of ζ chain expression (Fig. 1*C*). Taken together, we conclude that T lymphocytes localized to the spleen of *P. gingivalis*-treated mice are affected by the splenic immunosuppressive environment and reduce their ζ chain expression levels. These cells can exit the spleen and migrate to the lymph nodes, where recovery of ζ expression is allowed. It is important to note that cells passing through the peripheral blood of the treated mice, which is characterized by elevated levels of $Gr1^+Mac1^+$, maintain low levels of ζ expression until reaching the lymph nodes, where they recover.

Discussion

In this study we describe the intricate effect of chronic inflammatory responses on TCR ζ chain expression and T cell function in different lymphatic organs. Herein we used an in vivo mouse experimental system (15), which is characterized by chronic

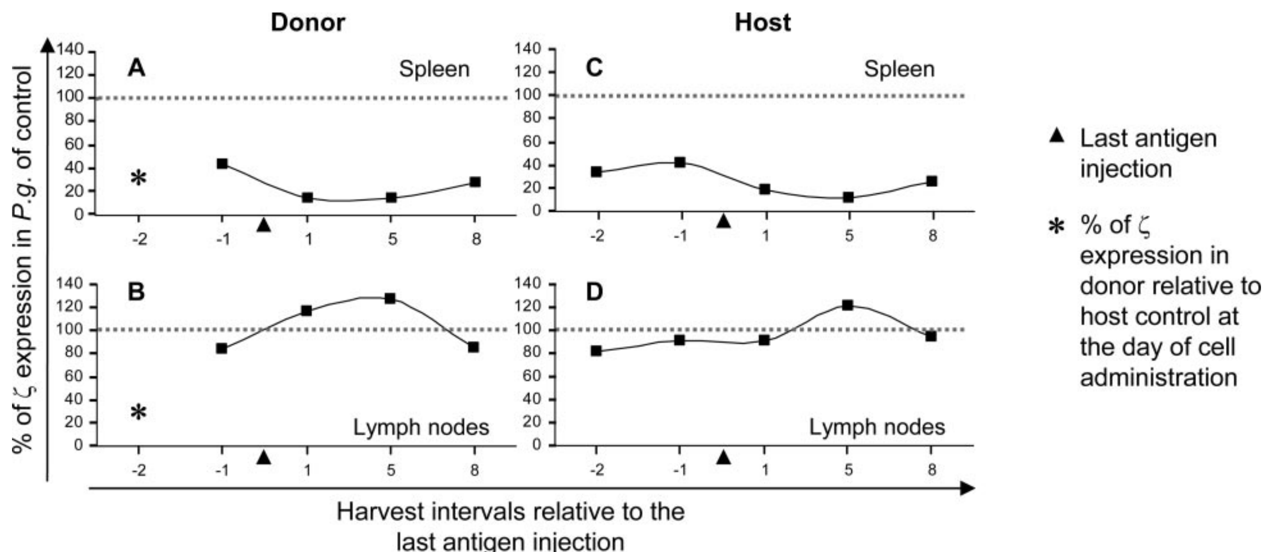

FIGURE 7. Adoptively transferred *P. gingivalis*-T cells are differentially regulated in the spleen and lymph nodes of mice with chronic inflammation. PKH-26 labeled splenocytes derived from *P. gingivalis*-treated mice (donor cells) were injected into the spleen of *P. gingivalis*-treated or control mice (hosts). After 24 h and at the indicated time points, spleen and lymph nodes of the host were harvested, and cell suspension was double stained for Thy1.2 and ζ expression. The ζ expression levels were detected in T cells that were found within the PKH-26 $^+$ (donor *A* and *B*) or PKH-26$^-$ population (host *C* and *D*). ζ expression of donor cells localized within the control mice (*A* and *B*) and of the hosts' control mice (*C* and *D*) were designed as 100% (discontinuous gray lines). Accordingly, ζ expression levels of the donor cells localized within *P. gingivalis*-treated mice and of the *P. gingivalis*-treated host cells are presented as percentage of ζ expression in *P. gingivalis* of control and calculated for each day (■) in spleen (*A* and *C*) and lymph nodes (*B* and *D*), as described in Fig. 6. (*) asterisks represent the percentage of ζ expression of the donor cells relative to the control at the time of administration. In this figure, cells from *P. gingivalis*-treated mice expressing 34% of ζ from the control mice were injected into the different hosts.

inflammation associated with immunosuppression. A bystander immunosuppressive effect was observed, as indicated by down-regulation of TCR ζ chain expression and impaired T cell function in the entire splenic T cell population. Our cumulative data (15) indicate that this experimental system mimics the conditions generated in pathologies such as cancer, autoimmune, and infectious diseases, all of which are characterized by abnormal T cell function and impaired TCR ζ expression. The negative effect of the chronic inflammatory immune response on T cells within the spleen is time-limited and reversible, since upon withdrawal of the Ag ζ chain expression levels in the spleen T cells began to recover by day 8–12 and eventually returned to normal.

Surprisingly, the results presented herein show that the effects of chronic inflammation on T cells are organ dependent. Comparison between the characteristics of T cells in the lymph nodes and spleen of the treated mice revealed that, in contrast to the abnormal characteristics of splenic T cells, T cells isolated from the lymph nodes of the same mice exhibit normal features, as indicated by normal TCR ζ expression and TCR-mediated functions. Thus, in mice exhibiting a chronic inflammatory immune response, resident T cells in various lymphatic organs are differentially affected. Although both lymph nodes and spleens of the treated mice are enlarged, as expected due to the excessive antigenic exposure, only the spleen displays an abnormal morphology/structure. We show that the follicular organization of the spleen from treated mice is disturbed. Moreover, T cells are absent from the white pulp zone and are found only within the red pulp zone. This is in contrast to the normal distribution of T cells between both zones in the spleen of control mice. The compartmentalized structures of the spleen and lymph nodes control cellular interactions that are required for the execution of an optimal response (25). Thus, the abnormal T cell distribution within the spleen is expected to affect T cell function. Indeed, the abnormalities of spleen morphology and structure in the treated mice correlate with the observed ζ down-regulation and T cell malfunction. This is in contrast to the normal follicular structure of the lymph nodes detected in the treated mice that correlates with normal TCR ζ expression and T cell function. The specific phenotypic differences between T cells in the spleen of the treated mice and those of control mice that could affect their localization in the white pulp zone are yet to be identified.

Exclusion of T cells from the spleen white pulp was shown in mice that were immunized with OVA peptides combined with complete Freund's or CpG oligonucleotides as adjuvants, but not following immunization with the full OVA protein (26). In the described study, only the specific-antigenic T cells were analyzed and the exclusion of T cells from the white pulp was attributed to the suboptimal activation induced upon peptide immunization (26). Another study demonstrated that exposure to pertussis toxin alters migration of T and B cells in the spleen, blocking their entrance to the white pulp zone. However, in that study, the failure of pertussis toxin-treated lymphocytes to enter the white pulp corresponded with their failure to enter the lymph nodes as well (16). This is in contrast to our findings that T cells in *P. gingivalis*-treated mice are functionally suppressed in the spleen and are excluded from the white pulp, while in the lymph nodes they are present, and exhibit normal characteristics.

The observed differences between T cells in the spleen and lymph nodes of the treated mice suggest that two distinct immunological environments exist in the different lymphatic organs. Indeed, analysis of the cellular composition of the spleen and lymph nodes of the treated mice revealed that they differ in their cellular profile. The number of the $Gr1^+Mac\text{-}1^+$ double-positive cells increased significantly in the spleens of the treated mice and was hardly detected in the lymph nodes of the same mice, or in the spleen and lymph nodes of control mice. Moreover, elevated numbers of these cells were detected as well in the peripheral blood and bone marrow of the treated mice. These changes in cellular composition correlate with ζ expression levels and in vitro activity of T cells obtained from these organs. Although T cells in the spleen of *P. gingivalis*-treated mice expressing partial TCR (ζ deficient) or low levels of ζ containing TCR could signal for the expression of normal levels of the cytokines IL-2 and IFN-γ, they express lower levels of IL-2R and show impaired in vitro proliferative response when compared with T cells from the spleen of control mice. Thus, the immunosuppressive milieu generated by the MSC in conjunction with ζ down-regulation lead to in vitro T cell dysfunction. Furthermore, we previously showed that *P. gingivalis*-treated mice infected with influenza virus clear the virus from the lungs less efficiently as compared with control mice (15), suggesting that the in vivo immune status of the *P. gingivalis*-treated mice is dictated by the immunosuppressive conditions in the bone marrow, spleen, and peripheral blood.

Herein we demonstrate that $Gr1^+Mac\text{-}1^+$ cells play a major role in the generation of the immunosuppressive conditions observed in the treated mice. These cells induce ζ down-regulation and impaired TCR-mediated function of normal T cells as demonstrated by ex vivo coincubation experiments. It is important to note that a low number of $Gr1^+Mac\text{-}1^+$ cells were also detected in the spleen of control mice and their ratio relative to T cells was 1:5. This is in contrast to the elevated number of $Gr1^+Mac\text{-}1^+$ cells in the spleen of the treated mice, where their ratio with T cells was 2:1. We demonstrate that $Gr1^+Mac\text{-}1^+$ cells isolated from control mice have the potential to induce ζ down-regulation if their ratio to T cells is increased to 2:1, as detected in the spleen of the treated mice. However, the induction of ζ down-regulation was less pronounced than that induced by the $Gr1^+Mac\text{-}1^+$ cells isolated from the spleen of the treated mice. The significance of the increased relative abundance of $Gr1^+Mac\text{-}1^+$ cells to T cells for imposing their immunosuppressive effect, is supported by our observations analyzing the bone marrow of normal mice. In the bone marrow of normal mice, where the basal level of $Gr1^+Mac\text{-}1^+$ cells is elevated and their ratio to T cells is 16:1, ζ expression levels are significantly reduced compared with those of normal splenic T cells, where the $Gr1^+Mac\text{-}1^+$ to T cell ratio is 1:10. Here again, the sole affected TCR subunit is the ζ chain. $Gr1^+Mac\text{-}1^+$ cells were also detected in the peripheral blood of the treated mice where their concentration is greater than that in the spleen, with a ratio of 3:1 $Gr1^+Mac\text{-}1^+$ cells vs T cells. However, although the levels of $Gr1^+Mac\text{-}1^+$ cells are increased in the peripheral blood, the degree of ζ down-regulation observed in T cells derived from PBL is similar to that found in splenic T cells. This discrepancy could be attributed to the inefficient contact between the $Gr1^+Mac\text{-}1^+$ cells and the T cells in the peripheral blood, in contrast to the effective interaction between the cells in the packed splenic zones. The later raises a yet unresolved question of how $Gr1^+Mac\text{-}1^+$ cells in the peripheral blood induce ζ down-regulation. As mentioned above, T cells in the spleen of the *P. gingivalis*-treated mice were localized to the red pulp zone, which is enriched with $Gr1^+$ cells (22). Moreover, we demonstrate that the percentage of $Gr1^+Mac\text{-}1^+$ cells in the spleen of mice exhibiting chronic inflammation increases tremendously, similar to that described in tumor-bearing hosts (27–30). Thus, the efficient immunosuppressive effect of $Gr1^+Mac\text{-}1^+$ cells on splenic T cells of the treated mice could be explained by 1) the changes in the cellular distribution, the elevated numbers and enhanced state of activation of $Gr1^+Mac\text{-}1^+$ cells in the spleen of the treated mice, 2) the requirements for contact and/or close proximity between $Gr1^+Mac\text{-}1^+$ cells and T cells to mediate the immunosuppressive

effect, and 3) yet uncharacterized changes in the $Gr1^+Mac\text{-}1^+$ cells affecting their immunosuppressive function. Our observations are supported by previous studies demonstrating that cell-cell contact is required for MSC, isolated from tumor-bearing hosts, to exert their immunosuppressive effect on T cells (28, 31, 32). Changes in the $Gr1^+Mac\text{-}1^+$ cells within the spleen of the treated mice could result from a massive recruitment of the $Gr1^+Mac\text{-}1^+$ cells from the bone marrow, followed by activation in the spleen and/or from a extensive in situ cell activation and proliferation (hemopoiesis). Our previous studies suggest that IFN-γ plays a major role in the generation/activation/recruitment of the $Gr1^+Mac\text{-}1^+$ cells that induce the immunosuppressive environment. IFN-γ deficient mice, when subjected to the *P. gingivalis* treatment described herein, do not develop an immunosuppressive environment; their T cells express normal levels of ζ chain and their immune function is not affected (15). In tumor models, GM-CSF was shown to play an important role in the recruitment/hemopoiesis of these myeloid cells to the spleen (28, 33). Disproportionate hemopoiesis, splenomegaly and abnormal immune function were shown as well in mice repetitively exposed to CpG (34, 35). However, this point remains to be clarified in the *P. gingivalis*-treated mouse model.

Mouse $Gr1^+Mac\text{-}1^+$ myeloid cells have a mixed mature and immature phenotype (23), and were shown to be responsible for inhibiting in vitro T cell proliferation upon TCR-mediated activation. $Gr1^+Mac\text{-}1^+$ cells in tumor-bearing hosts affect tumor-specific and nonspecific T cells (27, 28, 32, 36, 37). The immunosuppressive effect of $Gr1^+Mac\text{-}1^+$ cells on T cells is attributed primarily to the function of two enzymes that metabolize L-arginine, arginase, and NO synthase (38–40). The immunosuppressive effect of $Gr1^+Mac\text{-}1^+$ cells on T cells could be mediated by the activation of one or more pathways within T cells that lead to their impaired function, one of which could involve the induction of TCR ζ chain down-regulation by its targeting to lysosomal degradation, as shown in our previous report (15). Depletion of L-arginine from the medium was shown to down-regulate the TCR ζ chain expression in Jurkat T cells (41). Another study reported that L-arginine depletion by tumor-associated mature myeloid cells blocks the re-expression of TCR ζ and CD3ε upon T cell stimulation (42). These observations are in contrast to the various studies showing that the sole TCR subunit to be affected in the above-described pathologies that are characterized by chronic inflammation, including in our model system, is the TCR ζ chain. Thus, the mechanism by which activated $Gr1^+Mac\text{-}1^+$ cells confer their immunosuppressive effect on all T cells (activated and nonactivated), either under chronic inflammatory conditions as depicted by our system, or in tumor-bearing hosts, remains to be determined.

Our results demonstrate that hosts mounting a chronic inflammatory response display several immunological milieus that differently affect T cell characteristics and function. The immunosuppressive environments characterizing the spleen, peripheral blood and bone marrow dictate the general impaired immune status of hosts with chronic inflammation, similar to that observed in tumor-bearing hosts and in those with other pathologies. Thus, the immunosuppressive environment created in hosts suffering from chronic inflammation not only affects endogenous T cells, but is expected to affect any newly introduced T cells, as well. This issue is of great concern when planning various immunotherapeutic strategies, such as vaccination and T cell transfer used in cancer patients (43, 44). In cases where an immunosuppressive environment exists in affected hosts, a limited success of a given immunotherapy is anticipated. Indeed, our in vivo cell transfer experiments that mimic therapy often used in diverse pathologies, as those mentioned above, revealed that normal donor T cells transferred into *P. gingivalis*-treated mice significantly down-regulated TCR ζ chain expression when localized in the spleen, while cells localized to the lymph nodes were not affected. Thus, the features of normal T cells transferred into mice exhibiting a suppressive environment are affected and adopt the characteristics of the endogenous suppressed cells. Our results revealed as well that T cells expressing low levels of ζ expression in the spleen of the treated mice were able to exit the spleen, reach the lymph nodes and recover their ζ chain expression. These results indicate that the affected T cells have the potential to circulate and modify their ζ chain expression levels depending on the environment they reach. Accordingly, the lymph nodes in mice exhibiting chronic inflammatory response are most likely important in maintaining a pool of normal T cells required for preserving a basal level of immune function, which is critical under the immunosuppressive conditions dictated by the spleen, peripheral blood and bone marrow. Thus, it is most likely that the general immune status of hosts with chronic inflammation is impaired but not totally immunosuppressed. These results are critical for cases where T cell transfer therapy is considered. One would have to take into consideration the existence of an immunosuppressive environment and make efforts to counteract it before or in conjunction with the applied immunotherapy.

To summarize, we show that sustained exposure to Ag that induces chronic inflammatory conditions differentially affects various secondary lymphatic organs; while T cells in the lymph nodes remain functional and express normal ζ chain levels, cells in the spleen, peripheral blood and bone marrow are negatively affected and reduce ζ expression levels and their function is impaired. The major effectors in exerting the inhibitory environment under such chronic inflammatory conditions are the $Gr1^+Mac1^+$ MSC. The studies presented here and those of others describing accumulation of regulatory MSC in lymphoid organs during tumor growth, graft-vs-host reactions and some infectious diseases (24, 45–47), and their association with impaired T cell responses to TCR-mediated stimuli, strongly suggest that MSC play a key role in controlling T cell responses under chronic inflammatory conditions (48). Chronic inflammation can develop under "sterile conditions" as during tumor growth and autoimmune diseases or under "pathogenic conditions" as in any infectious disease (49). Efforts to elucidate the mechanisms responsible for the immunosuppressive effect of MSC on T cells, which develops under such conditions, and finding ways to neutralize them, will result in the improvement of T cell transfer and vaccine-based therapies applied today for the treatment of such pathologies.

Acknowledgments

We gratefully acknowledgment the support of the Society of Research Associates of the Lautenberg Center, the Concern Foundation of Los Angeles, and the Harold B. Abramson Chair in Immunology. Thanks to Dr. Shelley Schwarzbaum for the critical reading of the manuscript and making constructive suggestions.

Disclosures

The authors have no financial conflict of interest.

References

1. Riccobon, A., R. Gunelli, R. Ridolfi, F. De Paola, E. Flamini, M. Fiori, C. Saltutti, M. Petrini, L. Fiammenghi, M. Stefanelli, et al. 2004. Immunosuppression in renal cancer: differential expression of signal transduction molecules in tumor-infiltrating, near-tumor tissue, and peripheral blood lymphocytes. *Cancer Invest.* 22: 871–877.
2. Finke, J. H., A. H. Zea, J. Stanley, D. L. Longo, H. Mizoguchi, R. R. Tubbs, R. H. Wiltrout, J. J. O'Shea, S. Kudoh, E. Klein, et al. 1993. Loss of T-cell receptor zeta chain and p56lck in T-cells infiltrating human renal cell carcinoma. *Cancer Res.* 53: 5613–5616.

3. Nakagomi, H., M. Petersson, I. Magnusson, C. Juhlin, M. Matsuda, H. Mellstedt, J. L. Taupin, E. Vivier, P. Anderson, and R. Kiessling. 1993. Decreased expression of the signal-transducing ζ chains in tumor-infiltrating T-cells and NK cells of patients with colorectal carcinoma. *Cancer Res.* 53: 5610–5612.
4. Matsuda, M., M. Petersson, R. Lenkei, J. L. Taupin, I. Magnusson, H. Mellstedt, P. Anderson, and R. Kiessling. 1995. Alterations in the signal-transducing molecules of T cells and NK cells in colorectal tumor-infiltrating, gut mucosal and peripheral lymphocytes: correlation with the stage of the disease. *Int. J. Cancer* 61: 765–772.
5. Lai, P., H. Rabinowich, P. A. Crowley-Nowick, M. C. Bell, G. Mantovani, and T. L. Whiteside. 1996. Alterations in expression and function of signal-transducing proteins in tumor-associated T and natural killer cells in patients with ovarian carcinoma. *Clin. Cancer Res.* 2: 161–173.
6. Kono, K., M. E. Ressing, R. M. Brandt, C. J. Melief, R. K. Potkul, B. Andersson, M. Petersson, W. M. Kast, and R. Kiessling. 1996. Decreased expression of signal-transducing ζ chain in peripheral T cells and natural killer cells in patients with cervical cancer. *Clin. Cancer Res.* 2: 1825–1828.
7. Kurt, R. A., W. J. Urba, J. W. Smith, and D. D. Schoof. 1998. Peripheral T lymphocytes from women with breast cancer exhibit abnormal protein expression of several signaling molecules. *Int. J. Cancer* 78: 16–20.
8. Kuss, I., T. Saito, J. T. Johnson, and T. L. Whiteside. 1999. Clinical significance of decreased ζ chain expression in peripheral blood lymphocytes of patients with head and neck cancer. *Clin. Cancer Res.* 5: 329–334.
9. Healy, C. G., J. W. Simons, M. A. Carducci, T. L. DeWeese, M. Bartkowski, K. P. Tong, and W. E. Bolton. 1998. Impaired expression and function of signal-transducing ζ chains in peripheral T cells and natural killer cells in patients with prostate cancer. *Cytometry* 32: 109–119.
10. Trimble, L. A., and J. Lieberman. 1998. Circulating CD8 T lymphocytes in human immunodeficiency virus-infected individuals have impaired function and downmodulate CD3ζ, the signaling chain of the T-cell receptor complex. *Blood* 91: 585–594.
11. Zea, A. H., M. T. Ochoa, P. Ghosh, D. L. Longo, W. G. Alvord, L. Valderrama, R. Falabella, L. K. Harvey, N. Saravia, L. H. Moreno, and A. C. Ochoa. 1998. Changes in expression of signal transduction proteins in T lymphocytes of patients with leprosy. *Infect. Immun.* 66: 499–504.
12. Berg, L., J. Ronnelid, L. Klareskog, and A. Bucht. 2000. Down-regulation of the T cell receptor CD3 ζ chain in rheumatoid arthritis (RA) and its influence on T cell responsiveness. *Clin. Exp. Immunol.* 120: 174–182.
13. Maurice, M. M., A. C. Lankester, A. C. Bezemer, M. F. Geertsma, P. P. Tak, F. C. Breedveld, R. A. van Lier, and C. L. Verweij. 1997. Defective TCR-mediated signaling in synovial T cells in rheumatoid arthritis. *J. Immunol.* 159: 2973–2978.
14. Liossis, S. N., X. Z. Ding, G. J. Dennis, and G. C. Tsokos. 1998. Altered pattern of TCR/CD3-mediated protein-tyrosyl phosphorylation in T cells from patients with systemic lupus erythematosus: deficient expression of the T cell receptor ζ chain. *J. Clin. Invest.* 101: 1448–1457.
15. Bronstein-Sitton, N., L. Cohen-Daniel, I. Vaknin, A. V. Ezernitchi, B. Leshem, A. Halabi, Y. Houri-Hadad, E. Greenbaum, Z. Zakay-Rones, L. Shapira, and M. Baniyash. 2003. Sustained exposure to bacterial antigen induces interferon-γ-dependent T cell receptor ζ down-regulation and impaired T cell function. *Nat. Immunol.* 4: 957–964.
16. Genco, C. A., and R. J. Arko. 1994. Animal chamber models for study of host-parasite interactions. *Methods Enzymol.* 235: 120–140.
17. Houri-Haddad, Y., W. A. Soskolne, A. Halabi, V. Barak, and L. Shapira. 2000. Repeat bacterial challenge in a subcutaneous chamber model results in augmented tumour necrosis factor-α and interferon-γ response, and suppression of interleukin-10. *Immunology* 99: 215–220.
18. Kesavalu, L., J. L. Ebersole, R. L. Machen, and S. C. Holt. 1992. Porphyromonas gingivalis virulence in mice: induction of immunity to bacterial components. *Infect. Immun.* 60: 1455–1464.
19. Baker, P. J., R. T. Evans, and D. C. Roopenian. 1994. Oral infection with Porphyromonas gingivalis and induced alveolar bone loss in immunocompetent and severe combined immunodeficient mice. *Arch Oral Biol.* 39: 1035–1040.
20. Frolov, I., Y. Houri-Hadad, A. Soskolne, and L. Shapira. 1998. In vivo exposure to *Porphyromonas gingivalis* up-regulates nitric oxide but suppresses tumour necrosis factor-α production by cultured macrophages. *Immunology* 93: 323–328.
21. Cyster, J. G., and C. C. Goodnow. 1995. Pertussis toxin inhibits migration of B and T lymphocytes into splenic white pulp cords. *J. Exp. Med.* 182: 581–586.
22. Nolte, M. A., E. N. Hoen, A. van Stijn, G. Kraal, and R. E. Mebius. 2000. Isolation of the intact white pulp: quantitative and qualitative analysis of the cellular composition of the splenic compartments. *Eur. J. Immunol.* 30: 626–634.
23. Taylor, P. R., G. D. Brown, A. B. Geldhof, L. Martinez-Pomares, and S. Gordon. 2003. Pattern recognition receptors and differentiation antigens define murine myeloid cell heterogeneity ex vivo. *Eur. J. Immunol.* 33: 2090–2097.
24. Kusmartsev, S., and D. I. Gabrilovich. 2002. Immature myeloid cells and cancer-associated immune suppression. *Cancer Immunol. Immunother.* 51: 293–298.
25. Kuby J. 2002. *Immunology.* W. H Freeman, San Francisco.
26. Zhang, L., E. F. Lizzio, T. Chen, and S. Kozlowski. 2005. Peptide immunization excludes antigen-specific T cells from splenic lymphoid compartments. *Eur. J. Immunol.* 35: 776–785.
27. Kusmartsev, S. A., Y. Li, and S. H. Chen. 2000. Gr-1$^+$ myeloid cells derived from tumor-bearing mice inhibit primary T cell activation induced through CD3/CD28 costimulation. *J. Immunol.* 165: 779–785.
28. Bronte, V., D. B. Chappell, E. Apolloni, A. Cabrelle, M. Wang, P. Hwu, and N. P. Restifo. 1999. Unopposed production of granulocyte-macrophage colony-stimulating factor by tumors inhibits CD8$^+$ T cell responses by dysregulating antigen-presenting cell maturation. *J. Immunol.* 162: 5728–5737.
29. Bronte, V., E. Apolloni, A. Cabrelle, R. Ronca, P. Serafini, P. Zamboni, N. P. Restifo, and P. Zanovello. 2000. Identification of a CD11b$^+$/Gr-1$^+$/CD31$^+$ myeloid progenitor capable of activating or suppressing CD8$^+$ T cells. *Blood* 96: 3838–3846.
30. Li, Q., P. Y. Pan, P. Gu, D. Xu, and S. H. Chen. 2004. Role of immature myeloid Gr-1+ cells in the development of antitumor immunity. *Cancer Res.* 64: 1130–1139.
31. Terrazas, L. I., K. L. Walsh, D. Piskorska, E. McGuire, and D. A. Harn, Jr. 2001. The schistosome oligosaccharide lacto-*N*-neotetraose expands Gr1$^+$ cells that secrete anti-inflammatory cytokines and inhibit proliferation of naive CD4$^+$ cells: a potential mechanism for immune polarization in helminth infections. *J. Immunol.* 167: 5294–5303.
32. Serafini, P., I. Borrello, and V. Bronte. 2006. Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin. Cancer Biol.* 16: 53–65.
33. Tsuchiya, Y., M. Igarashi, R. Suzuki, and K. Kumagai. 1988. Production of colony-stimulating factor by tumor cells and the factor-mediated induction of suppressor cells. *J. Immunol.* 141: 699–708.
34. Sparwasser, T., L. Hultner, E. S. Koch, A. Luz, G. B. Lipford, and H. Wagner. 1999. Immunostimulatory CpG-oligodeoxynucleotides cause extramedullary murine hemopoiesis. *J. Immunol.* 162: 2368–2374.
35. Heikenwalder, M., M. Polymenidou, T. Junt, C. Sigurdson, H. Wagner, S. Akira, R. Zinkernagel, and A. Aguzzi. 2004. Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxynucleotide administration. *Nat. Med.* 10: 187–192.
36. Goni, O., P. Alcaide, and M. Fresno. 2002. Immunosuppression during acute *Trypanosoma cruzi* infection: involvement of Ly6G (Gr1$^+$)CD11b$^+$ immature myeloid suppressor cells. *Int. Immunol.* 14: 1125–1134.
37. Otsuji, M., Y. Kimura, T. Aoe, Y. Okamoto, and T. Saito. 1996. Oxidative stress by tumor-derived macrophages suppresses the expression of CD3 ζ chain of T-cell receptor complex and antigen-specific T-cell responses. *Proc. Natl. Acad. Sci. USA* 93: 13119–13124.
38. Bronte, V., M. Wang, W. W. Overwijk, D. R. Surman, F. Pericle, S. A. Rosenberg, and N. P. Restifo. 1998. Apoptotic death of CD8$^+$ T lymphocytes after immunization: induction of a suppressive population of Mac-1$^+$/Gr-1$^+$ cells. *J. Immunol.* 161: 5313–5320.
39. Wu, G., and S. M. Morris, Jr. 1998. Arginine metabolism: nitric oxide and beyond. *Biochem. J.* 336(Pt. 1): 1–17.
40. Bogdan, C. 2001. Nitric oxide and the immune response. *Nat. Immunol.* 2: 907–916.
41. Rodriguez, P. C., A. H. Zea, K. S. Culotta, J. Zabaleta, J. B. Ochoa, and A. C. Ochoa. 2002. Regulation of T cell receptor CD3ζ chain expression by L-arginine. *J. Biol. Chem.* 277: 21123–21129.
42. Rodriguez, P. C., D. G. Quiceno, J. Zabaleta, B. Ortiz, A. H. Zea, M. B. Piazuelo, A. Delgado, P. Correa, J. Brayer, E. M. Sotomayor, et al. 2004. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res.* 64: 5839–5849.
43. Rosenberg, S. A. 2004. Shedding light on immunotherapy for cancer. *N. Engl. J. Med.* 350: 1461–1463.
44. Abken, H., A. Hombach, and C. Heuser. 2003. Immune response manipulation: recombinant immunoreceptors endow T-cells with predefined specificity. *Curr. Pharm. Des.* 9: 1992–2001.
45. Gabrilovich, D. I., M. P. Velders, E. M. Sotomayor, and W. M. Kast. 2001. Mechanism of immune dysfunction in cancer mediated by immature Gr-1$^+$ myeloid cells. *J. Immunol.* 166: 5398–5406.
46. Bobe, P., K. Benihoud, D. Grandjon, P. Opolon, L. L. Pritchard, and R. Huchet. 1999. Nitric oxide mediation of active immunosuppression associated with graft-versus-host reaction. *Blood.* 94: 1028–1037.
47. Mencacci, A., C. Montagnoli, A. Bacci, E. Cenci, L. Pitzurra, A. Spreca, M. Kopf, A. H. Sharpe, and L. Romani. 2002. CD80$^+$Gr-1$^+$ myeloid cells inhibit development of antifungal Th1 immunity in mice with candidiasis. *J. Immunol.* 169: 3180–3190.
48. Baniyash, M. 2004. TCR ζ-chain downregulation: curtailing an excessive inflammatory immune response. *Nat. Rev. Immunol.* 4: 675–687.
49. Baniyash, M. 2006. Chronic inflammation, immunosuppression and cancer: New insights and outlook. *Semin. Cancer Biol.* 16: 80–88.