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Gr-1$^+$ Myeloid Cells Lacking T Cell Protein Tyrosine Phosphatase Inhibit Lymphocyte Proliferation by an IFN-γ- and Nitric Oxide-Dependent Mechanism[1]

Maryse Dupuis,* María de Jesús Ibarra-Sánchez,† Michel L. Tremblay,† and Pascale Duplay[2]*

The T cell protein tyrosine phosphatase is involved in the immune system regulation, as evidenced by defective function and development of several hemopoietic cell populations in T cell protein tyrosine phosphatase (TC-PTP)-deficient mice. In particular, B and T cell proliferation is greatly inhibited when total splenocytes are stimulated by LPS or anti-CD3 mAb. To define the functional defect of TC-PTP$^{-/-}$ lymphocytes, we isolated T and B cells from the spleen of TC-PTP$^{-/-}$ mice. We show that the proliferative response of lymphocytes was greatly increased when cultured as a purified population, indicating that an inhibitory population is present in TC-PTP$^{-/-}$ spleen. However, TC-PTP$^{-/-}$ lymphocytes have a 2- to 3-fold lower proliferation rate compared with TC-PTP$^{+/+}$ lymphocytes, suggesting that, as shown previously in embryonic fibroblasts, TC-PTP is involved in the control of cell cycle in lymphocytes. We have characterized phenotypically and functionally the inhibitory population present in the spleen of TC-PTP$^{-/-}$ mice. We show that a Gr-1$^+$-enriched cell population isolated from TC-PTP$^{-/-}$ mice suppresses the CD3-induced proliferation of T cells in coculture in vitro. The specific inhibition of NO synthesis with N^G-monomethyl-L-arginine.monoacetate restored splenocyte responses, and there is a strict correlation between NO levels and the degree of suppression. Neutralization of IFN-γ with specific mAb almost completely abolished the inhibitory activity of Gr-1$^+$ cells and concomitantly high levels of NO secretion. Moreover, inhibition of lymphocyte proliferative responses required cell-cell contact to achieve sufficient levels of NO. These findings demonstrate an important function of TC-PTP in the induction of the NO pathway that mediates inhibition of T cell proliferation. ***The Journal of Immunology,*** **2003, 171: 726–732.**

T cell protein tyrosine phosphatase (TC-PTP),[3] an intracellular tyrosine-specific protein phosphatase, is expressed ubiquitously (1). However, higher levels are found in hemopoietic tissues, suggesting that it plays an important role in the regulation of hemopoietic cell functions (1, 2). Indeed, homozygous null TC-PTP mutant animals show hemopoietic abnormalities manifested in splenomegaly, lymphoadenopathy, and loss of bone marrow cellularity (2). The bone marrow defect, most likely due to stroma cell deficiency, results in the failure to support the development of B cells and erythrocytes, but does not seem to affect myeloid development (2). T cell development appears also to be normal, as judged by normal numbers and ratio of CD4$^+$/CD8$^+$ cells both in the thymus and spleen (2).

In addition to its role in hemopoiesis, TC-PTP is involved in the regulation of lymphocyte functions because there is a specific defect in T and B cell proliferation of TC-PTP$^{-/-}$ splenocytes following Con A and LPS stimulation, respectively (2). Recently, TC-PTP has also been implicated in cytokine signaling through the negative regulation of Janus kinase 1 (Jak1) and Jak3 (3). Importantly, in response to IFN-γ, there is hyperphosphorylation of Jak1 and elevated expression of inducible NO synthase (iNOS) in bone marrow-derived macrophages from TC-PTP$^{-/-}$ mice. Because NO has been shown to be an important mediator of T cell suppression by activated macrophages (4–8), it is possible that the inhibition of T and B cell proliferation in response to activation signal is mediated by TC-PTP$^{-/-}$ macrophages that are likely to be hypersensitive to IFN-γ. Alternatively, but not exclusively, TC-PTP might play an important role in regulating lymphocyte activation. In murine embryonic fibroblasts, TC-PTP is a positive regulator of proliferation for the progression of G_1 phase of the cell cycle through the NF-κB pathway (9). It is therefore possible that TC-PTP has the same function in lymphocytes. This might explain, at least in part, the proliferation defect of TC-PTP$^{-/-}$ splenocytes.

In the present study, we examined whether the TC-PTP is involved in the regulation of lymphocyte and/or macrophage signaling. Our results clearly demonstrate that although TC-PTP$^{-/-}$ lymphocytes have a reduced proliferation rate compared with TC-PTP$^{+/+}$ lymphocytes, there is a cell population in the spleen of TC-PTP$^{-/-}$ mice that inhibits proliferative response induced by CD3 or B cell receptor ligation. Because Gr-1$^+$ myeloid cells have been shown to be involved in the suppression of lymphocyte activation (10–16), we purified these cells and studied their involvement in the regulatory mechanism of T and B cell activation in the TC-PTP$^{-/-}$ background. We found that NO induced by IFN-γ is the inhibitory effector of the TC-PTP$^{-/-}$ lymphocyte proliferation.

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[3] Abbreviations used in this paper: TC-PTP, T cell protein tyrosine phosphatase; iNOS, inducible NO synthase; Jak, Janus kinase; LME, L-leucine methyl ester; L-NMMA, N^G-monomethyl-L-arginine.monoacetate.

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Materials and Methods

Mice

TC-PTP-deficient mice of BALB/c background were previously described (2). Two- to 3-wk-old TC-PTP$^{-/-}$ and TC-PTP$^{+/+}$ mice were used in this study.

Cell preparation

Single-cell suspensions were prepared from spleens. RBC were removed by incubating the cells 5 min in lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA, pH 7.2), and the cell suspensions were passed through a 30-μM nylon mesh. T, B, and Gr-1$^+$ cells were obtained by sequential purification using anti-CD90 microbeads, anti-CD19 microbeads, and biotinylated anti-Gr-1 mAb (0.5 μg mAb/10^6 cells; eBioscience, San Diego, CA) plus streptavidin microbeads (Miltenyi Biotec, Auburn, CA), respectively. Positive and negative cell populations were separated on a MACS column, according to the manufacturer's instructions (Miltenyi Biotec). The last negative fraction, which corresponds to splenocytes depleted of T, B, and Gr-1$^+$ cells, is called Fr.3. Purity of the various populations was determined by flow cytometry analysis and was always 80% for T cells, 95% for B cells, and varied from 64 to 75% for Gr-1$^+$ cells.

Proliferation assays

Splenocytes (4×10^5 cells/well) were stimulated with 25 μg/ml plate-bound anti-CD3ϵ mAb (145.2C11) or with 10 μg/ml of LPS (Sigma-Aldrich, St. Louis, MO). Assays were performed in 96-well plates, 200 μl/well in RPMI 1640 medium containing 10% FBS, 50 μM 2-ME (Sigma-Aldrich), glutamine, antibiotics, 10 mM HEPES, pH 7.3, and 0.1 mM nonessential amino acid (Invitrogen, Burlington, Ontario, Canada). In all cocultures, an equal number of Gr-1$^+$ cells (1×10^5 cells) was added to purified T and B cells (1×10^5 cells). Cells were cultured for 24 h at 37°C, then pulsed with 2 μCi/ml [^{3}H]thymidine (ICN Biomedicals, Costa Mesa, CA) for an additional 18 h. Data are expressed as cpm (mean ± SD of triplicate cultures). Where indicated, IFN-γ at 100 U/ml (R&D Systems, Minneapolis, MN), 0.5 mM N^G-monomethyl-L-arginine.monoacetate (L-NMMA) (Alexis, San Diego, CA), or mouse rIL-2 (Roche Diagnostics, Laval, Canada) was added to cultures. Neutralizing anti-mouse IFN-γ mAb (R4-6A2) was used at 100 μg/ml. In some experiments, splenocytes were treated with L-leucine methyl ester (Sigma-Aldrich), as described previously (17). Some experiments were performed in 24-well transwell cell culture insert (0.4 μm pore; Costar, Cambridge, MA) in a final volume of 800 μl. T cells (5×10^5 cells) were in the lower compartment of the well, and Gr-1$^+$ cells (5×10^5 cells) were in the upper compartment. T cells were stimulated with 25 μg/ml plate-bound anti-CD3ϵ mAb (145.2C11). Where indicated, IFN-γ (100 U/ml; R&D Systems) was added to cultures. The same numbers of cells were cultured together in the lower compartment in 24-well plates. Cells were cultured for 24 h at 37°C, then pulsed with 2 μCi/ml [^{3}H]thymidine (ICN Biomedicals) for an additional 18 h. Cells in the lower compartment were transferred to 96-well plates to be harvested and counted.

NO measurements

In proliferation assays, supernatants were harvested after 18 or 42 h of culture and levels of NO were measured as the nitrite concentration by mixing equal volumes of culture supernatants (100 μl) and Griess reagent (18). Nitrite concentrations were determined by comparing the absorbance values of the test samples with a standard curve generated by serial dilution of 200 μM sodium nitrite.

Flow cytometry

Cells were surface stained using the following mAbs: PE anti-CD8 (rat IgG2a; eBioscience), FITC anti-F4/80 (rat IgG2b; Serotec, Hornby, Ontario, Canada), PE anti-CD80 (Armenian hamster IgG; eBioscience), PE anti-Gr-1 (rat IgG2b; eBioscience), and FITC anti-CD11b (rat IgG2b; kindly provided by S. Lemieux, INRS-Institut Armand-Frappier, Laval, Quebec, Canada). Acquisition of cells was performed using an EPICS XL (Coulter Electronics, Hialeah, FL). Dead cells were gated out based on the vital colorant 7-amino-actinomycin D staining (BD PharMingen, Mississauga, Ontario, Canada).

Western blot analysis

Splenocytes (2×10^6 cells) were washed in RPMI 1640 and resuspended at 4×10^7 cells/ml in RPMI 1640. Cells were left unstimulated or stimulated with anti-CD3ϵ (145.2C11, 10 μg/ml) for 30 min at 4°C. Cells were washed in RPMI 1640 and resuspended at 4×10^7 cells/ml in RPMI 1640. Cells were left unstimulated or stimulated with goat anti-hamster (30 μg/ml; Jackson ImmunoResearch, West Grove, PA) for the time indicated. Cells were harvested and solubilized for 30 min at 4°C in 1% Nonidet P-40 containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM EGTA in the presence of inhibitors of proteases and phosphatases (10 μg/ml leupeptin and aprotinin, 1 mM Pefabloc-sc, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4). Immunoblotting was performed, as described previously (19).

Measurement of intracellular Ca^{2+}

Spleen cells were enriched in T cells by removing B cells with Cellect mouse T cell column (Cytovax Biotechnologies, Edmonton, Alberta, Canada). B cell-depleted splenocytes were washed twice with HBSS and incubated at 10^7 cells/ml with 3 μM Indo-1 (Molecular Probes, Eugene, OR) and 0.4 mg/ml Pluronic acid F-127 (Molecular Probes) for 30 min at room temperature. Cells were washed in HBSS and resuspended at 10^6 cells/ml, and Ca^{2+} mobilization was conducted on an EPICS ELITE ESP cell sorter (Coulter Electronics). Cells were stimulated with anti-CD3ϵ mAb (145.2C11, 5 μg/ml) for 2 min at 37°C, and goat anti-hamster mAb (30 μg/ml; Jackson ImmunoResearch) was added.

Results

T and B cells purified from TC-PTP$^{-/-}$ splenocytes proliferate in response to CD3 or LPS stimulation

It has been previously demonstrated that splenocytes isolated from TC-PTP$^{-/-}$ mice failed to proliferate in response to Con A and LPS (2). To define whether this defective proliferative response is due to an intrinsic defect of lymphocytes or to a suppressive population present in the spleen of TC-PTP$^{-/-}$ mice, we purified the T and B cell population and tested their capacity to proliferate in response to CD3 or LPS stimulation. As expected, we found a dramatic defective proliferative response of TC-PTP$^{-/-}$ splenocytes to CD3 stimulation. CD3-induced proliferation was >20 times lower than the response of cells from TC-PTP$^{+/+}$ control splenocytes, despite the fact that the percentage of T cells was equivalent (Fig. 1*A*). Moreover, IL-2 addition to TC-PTP$^{-/-}$ splenocytes did not restore proliferation (Fig. 1*B*), indicating that the defective signaling pathway, if any, is downstream of IL-2 secretion. When T lymphocytes were purified from TC-PTP$^{-/-}$ splenocytes, their CD3-induced proliferation was greatly increased (Fig. 1*A*). As found for T cells, B cells failed to proliferate in response to LPS when tested within the total spleen cell population, whereas their proliferation was restored when present as a purified population (Fig. 1*C*). However, TC-PTP$^{-/-}$ T or B cells have a ~2- to 3-fold lower proliferative response compared with the response of T or B cells purified from TC-PTP$^{+/+}$ splenocytes. This is most likely due to the positive regulatory role of TC-PTP in proliferation (9).

Early TCR signaling is not affected in TC-PTP$^{-/-}$ T cells

To test the possibility that TC-PTP$^{-/-}$ T cells have a defect in TCR signaling that will be translated by a low proliferation rate, we examined early signaling events of splenic T cells initiated in response to CD3 cross-linking. As shown in Fig. 2*A*, the pattern and intensity of protein tyrosine phosphorylation induced by CD3 stimulation are similar in T cells from TC-PTP$^{+/+}$ or TC-PTP$^{-/-}$ splenocytes. Moreover, CD3-induced calcium mobilization is not affected in TC-PTP$^{-/-}$ T cells (Fig. 2*B*). These results indicate that any potential defect in TC-PTP$^{-/-}$ T cells is downstream of tyrosine phosphorylation and calcium mobilization responses.

Gr-1$^+$ cells isolated from TC-PTP$^{-/-}$ splenocytes inhibit T cell proliferation

To identify the suppressive cell population present in the TC-PTP$^{-/-}$ spleen, we used magnetic beads sorting. Because CD11b$^+$/Gr-1$^+$ myeloid cells have the ability to inhibit T cell

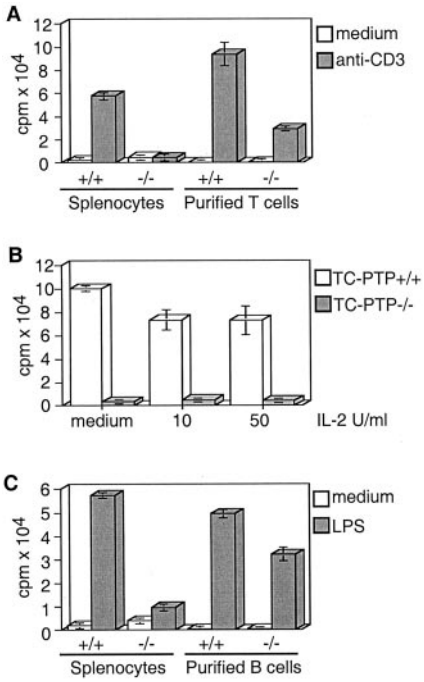

FIGURE 1. Purified T and B cells from TC-PTP$^{-/-}$ splenocytes proliferate after mitogen stimulation. *A*, Splenocytes (4×10^5) and purified T cells (2×10^5) from spleen of TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ mice were left unstimulated (medium) or stimulated on mAb-coated plate with anti-CD3. After 24 h, cells were pulsed with [^{3}H]thymidine and harvested 18 h later. Data represent means ± SD of triplicate cultures and are representative of 10 experiments. In the experiment shown, the percentage of CD3$^+$ and B220$^+$ cells was, respectively, 20 and 63% for TC-PTP$^{+/+}$ and 19 and 60% for TC-PTP$^{-/-}$ splenocytes. *B*, Splenocytes (4×10^5) from spleen of TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ mice were stimulated on mAb-coated plate with anti-CD3 in absence (−) or presence of IL-2, as indicated. Data represent means ± SD of triplicate cultures and are representative of two separate experiments. In the experiment shown, the percentage of CD3$^+$ cells was, respectively, 12% for TC-PTP$^{+/+}$ and 22% for TC-PTP$^{-/-}$ splenocytes. *C*, Splenocytes (4×10^5) and B cells (2×10^5) from spleen of TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ mice were left unstimulated (medium) or stimulated with LPS. The percentage of CD3$^+$ and B220$^+$ cells was as in *A*. After 24 h, cells were pulsed with [^{3}H]thymidine and harvested 18 h later.

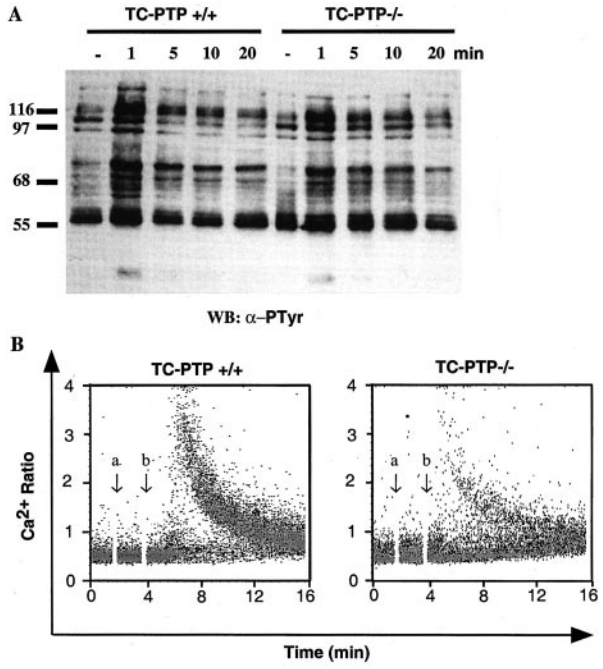

FIGURE 2. CD3-induced tyrosine phosphorylation and calcium response are not affected in T cells from TC-PTP$^{-/-}$ mice. *A*, Total cell lysates from T cells purified from splenocytes of TC-PTP$^{+/+}$ or TC-PTP$^{-/-}$ mice were analyzed by phosphotyrosine immunoblotting. Cells were left unstimulated (−) or stimulated with anti-CD3 mAb (145.2C11) for indicated time. *Left*, Positions of molecular mass markers are shown in kilodaltons. *B*, T cell-enriched splenocytes isolated from TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ mice were loaded with Indo-1 and stimulated with anti-CD3 (*arrow a*) and goat anti-hamster (*arrow b*) at the indicated time. Intracellular calcium was measured by comparing the ratio of Indo-1 emission at 381 nm (calcium-bound Indo-1) and 525 nm (free Indo-1) with excitation at 325 nm.

function (10–16, 20–22), we first asked whether the Gr-1$^+$ population in the spleen of TC-PTP$^{-/-}$ mice was responsible for the observed inhibition of T cell proliferation. TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ splenocytes were positively and sequentially purified for Thy-1 (T cells), CD19 (B cells), and Gr-1 (macrophages, NK, neutrophils) cell surface expression. Gr-1$^+$ and cells depleted in T, B, and Gr-1$^+$ were tested for their capacity to inhibit T cell proliferation. We used T cells isolated from TC-PTP$^{+/+}$ mice in these experiments to ensure that inhibitory activities are solely due to the tested population and avoid potential additional inhibitory effect resulting from the absence of TC-PTP in the TC-PTP$^{-/-}$ lymphocytes. Our results demonstrate that T cell proliferation after CD3 stimulation is completely abolished when T cells are cocultured with positively magnetic beads sorted Gr-1$^+$ cells from TC-PTP$^{-/-}$ splenocytes, whereas the remaining cells depleted of Gr-1$^+$ had no effect (Fig. 3*A*). Importantly, the same Gr-1$^+$ cell population from TC-PTP$^{+/+}$ did not inhibit cell proliferation when added to T cells (Fig. 3*A*). Moreover, the inhibitory Gr-1$^+$ cell population is sensitive to L-leucine methyl ester (LME) because treatment of TC-PTP$^{-/-}$ splenocytes with LME restored CD3-induced T cell proliferation (Fig. 3*C*). Because NO is known to be involved in immunosuppression (4–8, 23), we tested whether it plays a role in the inhibition of lymphocyte proliferation mediated by TC-PTP$^{-/-}$ Gr-1$^+$ cells. The NO production correlated with the intensity of the inhibition (Fig. 3*B*). Altogether these results demonstrate that a Gr-1$^+$ population present in the spleen of TC-PTP$^{-/-}$ mice inhibits T cell proliferation most likely by a NO-dependent mechanism.

Inhibition of T cell proliferation mediated by Gr-1$^+$ splenocytes from TC-PTP$^{-/-}$ mice is NO dependent and requires IFN-γ

To confirm that NO produced by Gr-1$^+$ TC-PTP$^{-/-}$ cells was the main mechanism for the suppression of proliferation, we asked whether L-NMMA, a competitive inhibitor of iNOS, was able to restore proliferation. The inhibition of proliferation could be completely reversed when L-NMMA was added to TC-PTP$^{+/+}$ T cells cocultured with TC-PTP$^{-/-}$ Gr-1$^+$ cells (Fig. 4*B*). As shown in Fig. 4*A*, although there is the same number of T cells in TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ splenocytes, the CD3-induced proliferation of TC-PTP$^{-/-}$ splenocytes was reduced in presence of L-NMMA compared with TC-PTP$^{+/+}$ splenocytes. As mentioned above, this is most likely due to the defective proliferation of TC-PTP$^{-/-}$ T cells. Moreover, the production of NO correlated with the levels of inhibition and was dependent of IFN-γ because a blocking anti-IFN-γ mAb prevented NO production and reversed completely the inhibition of proliferation mediated by Gr-1$^+$ cells (Fig. 4, *C* and *D*). These results suggest that NO production is dependent of IFN-γ and is involved in Gr-1$^+$ cell-mediated inhibition of T cell responses in TC-PTP$^{-/-}$ mice. This NO-mediated

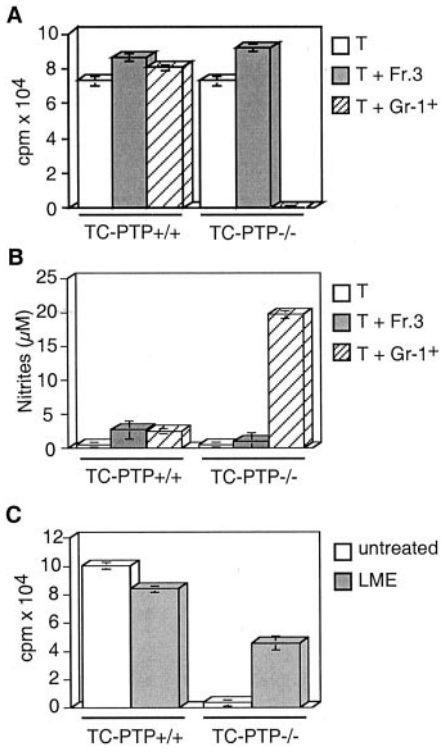

FIGURE 3. Gr-1$^+$ cells purified from splenocytes of TC-PTP$^{-/-}$ mice inhibit T cell proliferation. *A*, Purified T cells from TC-PTP$^{+/+}$ splenocytes were stimulated on mAb-coated plate with anti-CD3 with no additional cells (T) and in presence of the Fr.3 (T + Fr.3) or Gr-1$^+$ (T + Gr-1$^+$) cell population isolated from TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ mice. Gr-1$^+$ cells were purified with magnetic beads, and Fr.3 represents splenocytes depleted of T, B, and Gr-1$^+$ cells. The proliferation was measured by [^{3}H]thymidine incorporation, as described in Fig. 1. *B*, After 42 h, supernatants were harvested, and the amount of NO secreted was detected using Griess reagent. Data are the average of triplicate wells and are representative of four separate experiments. *C*, Splenocytes (4×10^5) from TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ mice were treated (LME) or untreated with L-leucine-methyl ester, and they were stimulated on mAb-coated plate with anti-CD3. After 24 h, cells were pulsed with [^{3}H]thymidine and harvested 18 h later. Data represent means ± SD of triplicate cultures and are representative of two experiments.

antiproliferative effect is partially reversed when T cells have been preactivated with anti-CD3 mAbs for 6 h prior to contact with the Gr-1$^+$ cells (Fig. 5).

Gr-1$^+$ cells from TC-PTP$^{-/-}$ mice inhibit T cell proliferation through a contact-dependent mechanism

We noticed repeatedly that Gr-1$^+$-enriched cells from TC-PTP$^{-/-}$, but not from TC-PTP$^{+/+}$ mice stimulated by IFN-γ added exogenously at 100 U/ml produced approximately one-half the amount of NO as when cultured with CD3-stimulated T cells (Fig. 6*A*). By contrast, resting T cells did not enhance the IFN-γ-induced NO production of Gr-1$^+$ TC-PTP$^{-/-}$ cells (Fig. 6*B*). These data suggest that additional signals mediated by activated T cells are required to achieve high levels of NO production by Gr-1$^+$ cells isolated from TC-PTP$^{-/-}$ spleen. To determine whether these signals involve cell-cell contact or only soluble mediators, we either cocultured the Gr-1$^+$-enriched cells with the T cells in the same well or separated by a semipermeable membrane. Suppression of CD3-induced proliferation occurred only if TC-PTP$^{-/-}$ Gr-1$^+$ cells were in contact with T lymphocytes (Fig. 6*C*). Similarly, high levels of NO production required a contact between

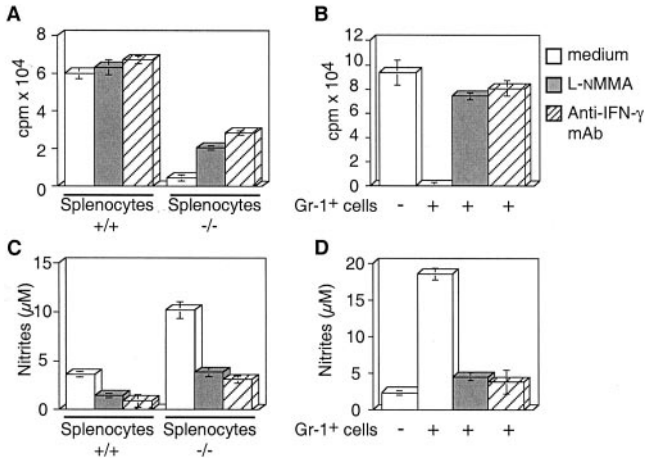

FIGURE 4. Involvement of IFN-γ and NO secretion in Gr-1$^+$ immune suppression. *A*, Splenocytes (T and B cell numbers as in Fig. 1) from TC-PTP$^{-/-}$ and TC-PTP$^{+/+}$ mice were stimulated on mAb-coated plate with anti-CD3 alone (medium) or in presence of L-NMMA (0.5 mM), a competitive inhibitor of iNOS, or a neutralizing anti-IFN-γ mAb (100 μg/ml). The proliferation was measured by [^{3}H]thymidine incorporation, as described in Fig. 1. *B*, Purified T cells from TC-PTP$^{+/+}$ splenocytes were stimulated with anti-CD3 and incubated with Gr-1$^+$ cells isolated from TC-PTP$^{-/-}$ spleen when indicated (+) and were treated as in *A*. The proliferation was measured by [^{3}H]thymidine incorporation, as described in Fig. 1. After 42 h, supernatants were harvested, and the amount of NO secreted onto the culture supernatants was detected using Griess reagent (*C* and *D*). Data are the average of triplicate wells and are representative of three separate experiments.

the Gr-1$^+$ cell population and T lymphocytes even in the presence of high amount of IFN-γ added exogenously (Fig. 6*D*).

Characterization of the Gr-1$^+$ cell population isolated from TC-PTP$^{-/-}$ mice

Suppression of immune responses in pathological or nonpathological situations results often from the accumulation of CD11b$^+$/Gr-1$^+$-suppressive myeloid cell populations in the spleen (10, 20). By contrast, flow cytometry analysis revealed that there was not an accumulation of CD11b$^+$/Gr-1$^+$ in the spleen of TC-PTP$^{-/-}$ mice (Fig. 7). To further characterize functionally the Gr-1$^+$ population, we tested whether secretion of NO was differently regulated in cells isolated from TC-PTP$^{-/-}$ than in those isolated from TC-PTP$^{+/+}$ splenocytes. As shown in Fig. 8*A*, LPS alone or IFN-γ alone induced NO production in TC-PTP$^{-/-}$ Gr-1$^+$ cells, but only when added in combination in TC-PTP$^{+/+}$ Gr-1$^+$ cells. These results indicate that TC-PTP$^{-/-}$ Gr-1$^+$ cells are in a different state of activation than TC-PTP$^{+/+}$ Gr-1$^+$ cells. In an attempt to characterize further the state of activation of Gr-1$^+$ cells, we compared their B7.1 (CD80) expression. The expression of B7.1, a marker that is up-regulated in B cells and macrophages when activated, was present in higher levels at the cell surface of unstimulated Gr-1$^+$ cells from TC-PTP$^{-/-}$ mice than Gr-1$^+$ cells from TC-PTP$^{+/+}$ mice (Fig. 8*B*). By contrast, there was not a significant difference in the percentage and level of expression of Gr-1$^+$, CD11b$^+$, or F4/80$^+$ cells between TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ Gr-1$^+$ population (Fig. 8*C*). The Gr-1$^+$ inhibitory cell population, i.e., Gr-1$^+$-purified cells depleted for T and B cells, is >83% CD11b$^+$ double positive (Fig. 8*C*). Altogether, these results demonstrate that there is in the spleen of TC-PTP$^{-/-}$ mice a Gr-1$^+$ cell population with the functional and phenotypic characteristics of activated cells.

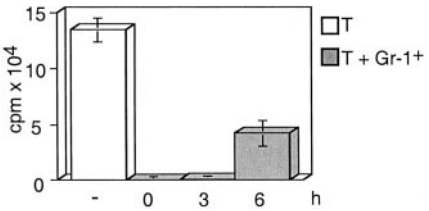

FIGURE 5. Preactivation of T cells prevents the inhibition of their proliferation by Gr-1$^+$ cells from TC-PTP$^{-/-}$ mice. Purified T cells (2×10^5) from TC-PTP$^{+/+}$ mice were stimulated with anti-CD3 alone (T) or in presence of Gr-1$^+$ cells (2×10^5) from TC-PTP$^{-/-}$ mice (T + Gr-1$^+$) after indicated time of preactivation on mAb-coated plate with anti-CD3. After 24 h, cells were pulsed with [^{3}H]thymidine and harvested 18 h later. Data represent means ± SD of triplicate cultures and are representative of two experiments.

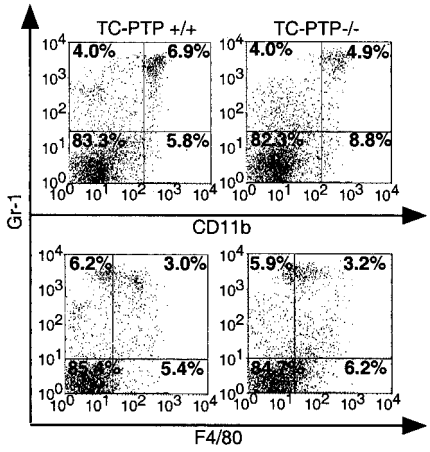

FIGURE 7. Gr-1$^+$ cells do not accumulate in the spleen of TC-PTP$^{-/-}$ mice. Flow cytometry analysis of the Gr-1$^+$ cell population from the spleen of TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ mice. Splenocytes were stained with FITC anti-CD11b and PE anti-Gr-1 mAbs or FITC anti-F4/80 and PE anti-Gr-1 mAbs. In each plot, 10,000 events were analyzed. The experiment was repeated five times with similar results.

Discussion

In this study, we have identified the cell population and examined the mechanism that leads to deficient proliferative responses to CD3 or LPS stimulation of TC-PTP$^{-/-}$ spleen cells. Our results clearly demonstrate that an inhibitory Gr-1$^+$ cell population in the spleen of TC-PTP-deficient mice prevents the proliferation of lymphocytes. This inhibitory activity is not linked to a phenotypic or functional defect of TC-PTP$^{-/-}$ lymphocytes because proliferation of TC-PTP$^{+/+}$ lymphocytes is inhibited to the same extent.

This inhibition is most likely mediated by NO because L-NMMA, an iNOS inhibitor, completely restores proliferation. Moreover, there is a strict correlation between NO levels and the degree of suppression. In myeloid cells, NO is produced by the inducible enzyme, iNOS, during the conversion of L-arginine to L-citrulline (24, 25). iNOS message and protein are induced by a variety of stimuli such as IFN-γ, TNF-α, IL-2, and LPS in macrophages (25–27). In our study, IFN-γ, produced by anti-CD3-activated T cells, is required for Gr-1$^+$ cell-mediated suppressive activity. Indeed, neutralization of IFN-γ with a specific mAb almost completely abolished the inhibitory activity of Gr-1$^+$ cells and concomitantly high levels of NO secretion. Moreover, IFN-γ-induced NO production required cell-cell contact to achieve the levels of NO sufficient for inhibition of lymphocyte proliferative responses. This cell-mediated signal is present on activated T cells, but not on resting cells. Moreover, CD3 stimulation of T lymphocytes 6 h before coculture with Gr-1$^+$ cells from TC-PTP$^{-/-}$ mice rescued the proliferation of the cells almost completely. Therefore,

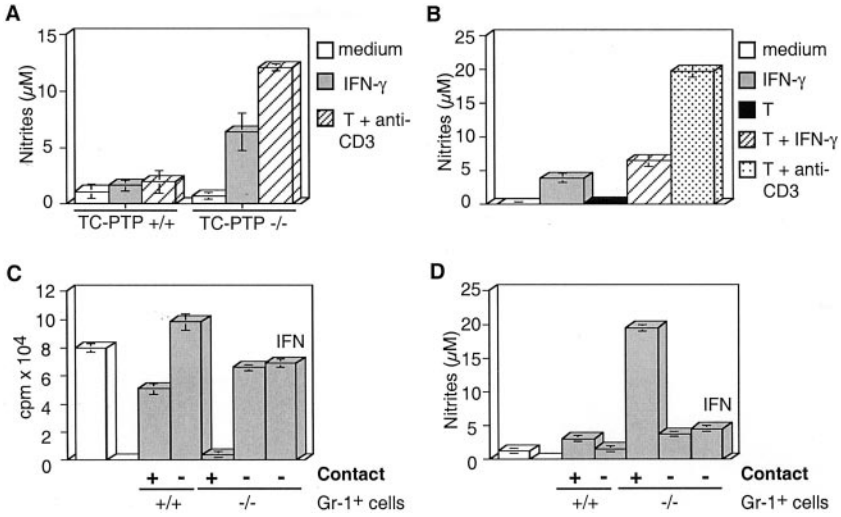

FIGURE 6. Suppression of T cell proliferation by Gr-1$^+$ cells purified from TC-PTP$^{-/-}$ splenocytes requires a cell-cell contact. *A*, Purified Gr-1$^+$ cells (1×10^5) from TC-PTP$^{+/+}$ splenocytes or TC-PTP$^{-/-}$ splenocytes were cultured alone (medium), with IFN-γ, or with 1×10^5 CD3-activated T cells isolated from TC-PTP$^{+/+}$ splenocytes (T + anti-CD3). After 18 h, supernatants were harvested, and the amount of NO secreted was detected using Griess reagent. *B*, Purified Gr-1$^+$ cells (1×10^5) from TC-PTP$^{-/-}$ mice were cultured alone (medium), with IFN-γ, with unstimulated purified T cells from TC-PTP$^{+/+}$ mice (T), or with CD3-stimulated purified T cells from TC-PTP$^{+/+}$ mice (T + anti-CD3). After 42 h, supernatants were harvested, and the amount of NO secreted was detected using Griess reagent. *C*, Purified T cells (5×10^5) from TC-PTP$^{+/+}$ splenocytes (T) were stimulated on mAb-coated plate with anti-CD3. T cells were cultured alone (open columns) or with Gr-1$^+$ cells (filled columns) purified from TC-PTP$^{-/-}$ or TC-PTP$^{+/+}$ splenocytes, as indicated. Where indicated, T cells were cultured with Gr-1$^+$ cells in the same well (+) or in separate compartments (−) of a transwell plate. IFN-γ was added in the upper compartment, where indicated (IFN). The proliferation was measured by [^{3}H]thymidine incorporation, as decribed in Fig. 1. *D*, After 42 h, supernatants were harvested, and the amount of NO secreted was detected using Griess reagent. Data were the average of triplicates and were confirmed by one other independently performed experiment.

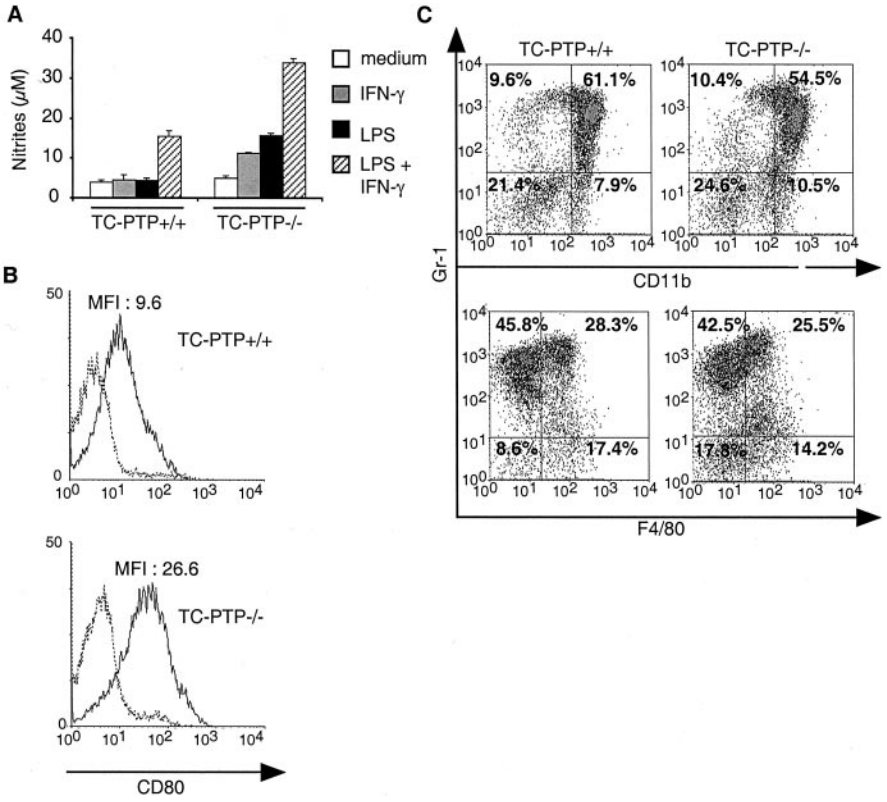

FIGURE 8. Functional and phenotypic characterization of Gr-1$^+$ cells isolated from TC-PTP$^{-/-}$ spleen. *A*, Gr-1$^+$ cells (2×10^5) purified from TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ splenocytes were left unstimulated (−) or were stimulated with 100 U/ml IFN-γ, 10 μg/ml LPS, or IFN-γ and LPS, as indicated. After 42-h culture, supernatants were harvested, and the amount of NO secreted was detected using Griess reagent. *B*, Flow cytometry analysis of B7.1 (CD80) expression at the cell surface of TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ Gr-1$^+$ cells. Gr-1$^+$ cells were stained with PE anti-B7.1 mAb (thick line). PE anti-CD8 were used as controls for background staining (dotted line). *C*, Flow cytometry analysis of purified Gr-1$^+$ cells from spleen of TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ mice. Purified Gr-1$^+$ cells were stained with FITC anti-CD11b and PE anti-Gr-1 mAbs or with FITC anti-F4/80 and PE anti-Gr-1 mAbs. In each plot, 10,000 events were analyzed. The experiment was repeated four times with similar results.

NO-mediated proliferative suppression most likely acts by preventing the cells from entering the cell cycle or arresting the cells before the first G_1/S transition. Additional experiments are required to identify the molecules involved in the increase of IFN-γ-induced NO secretion by Gr-1$^+$ cells, to characterize the cellular targets of NO, and to determine the effector mechanisms involved in the inhibition.

The Gr-1$^+$-inhibitory cell population in the spleen is heterogeneous and contains NK cells, macrophages, and neutrophils (28–30). There is not a significant difference in the percentage and level of expression of Gr-1$^+$, CD11b$^+$, or F4/80$^+$ cells between TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ Gr-1$^+$ populations. Depletion of the Gr-1$^+$ population of cells expressing the pan-NK marker DX5 did not abrogate inhibitory activity (data not shown). Moreover, treatment of splenocytes from TC-PTP$^{-/-}$ mice with LME, which kills selectively cells with high lysosomal content (17), restored T cell proliferation of splenocytes from TC-PTP$^{-/-}$ mice. Taken together, these results indicate that in the TC-PTP$^{-/-}$ spleen, the population of Gr-1$^+$ cells that inhibits T and B cell proliferation through an NO- and IFN-γ-dependent mechanism is likely to correspond to suppressor macrophages that express the granulocyte-monocyte markers CD11b and Gr-1, which have been studied in other experimental systems such as mice injected with superantigens, tumors, virus, cyclophosphamide, and bacteria (11–16, 21). This Gr-1$^+$-enriched population most likely corresponds to a heterogeneous population, and the cells responsible for the inhibition might correspond to a fraction of the Gr-1$^+$ CD11b$^+$ cells present in the spleen of TC-PTP$^{-/-}$ mice. The equivalent inhibitory cell population might be present in the TC-PTP$^{+/+}$ spleen, but most likely at much lower numbers. Moreover, as reflected by the increased expression of the cell surface marker B7.1, the Gr-1$^+$-enriched population from TC-PTP$^{-/-}$ mice had a more activated phenotype than TC-PTP$^{+/+}$ Gr-1$^+$ cells. Therefore, due to these quantitative and qualitative differences among TC-PTP$^{-/-}$ and TC-PTP$^{+/+}$ Gr-1$^+$ cells, much higher ratio of TC-PTP$^{+/+}$ Gr-1$^+$ cells will be required for efficient inhibition of T cell proliferation.

We demonstrate that Gr-1$^+$ cells from TC-PTP$^{-/-}$ mice display an altered sensitivity toward IFN-γ or LPS signals compared with Gr-1$^+$ cells from TC-PTP$^{+/+}$ mice. One possible explanation for the preactivation state of macrophages is an excess of IFN-γ secretion by T cells in vivo in TC-PTP$^{-/-}$ mice. Our experimental data support this hypothesis because, when stimulated with anti-CD3 mAb, TC-PTP$^{-/-}$ T cells produce larger amount of IFN-γ than TC-PTP$^{+/+}$ T cells (data not shown). Alternatively, altered development of these cells in the bone marrow of TC-PTP$^{-/-}$ mice might lead to the differentiation of these myeloid suppressor cells. The reported defect of stroma cells in the bone marrow of TC-PTP$^{-/-}$ mice might be responsible for the impaired development of myeloid cells (2). Moreover, TC-PTP has been shown to be a negative regulator of Jak1 and Jak3 (3), and the nuclear isoform of TC-PTP was identified as the Stat1 nuclear phosphatase (31, 32). Therefore, the expected high activity of Jak1 and Stat1 in TC-PTP$^{-/-}$ mice may explain, at least in part, the enhanced Stat1/Jak1/2-mediated response to IFN-γ and Stat1/Tyk2-mediated response to LPS in TC-PTP$^{-/-}$ macrophages. The hyperphosphorylation of Jak1 and elevated expression of iNOS of bone marrow-derived macrophage treated by IFN-γ support this hypothesis (3).

Moreover, our results indicate that in TC-PTP$^{-/-}$ T lymphocytes, early (i.e., calcium mobilization, tyrosine phosphorylation, and extracellular signal-regulated kinase 1/2 activation; Fig. 2 and data not shown) and late (i.e., IL-2 secretion; data not shown) TCR signaling events were not affected. These results are in agreement with studies showing that overexpression of TC-PTP does not affect IL-2 transcription (33). Nevertheless, it should be noted that, as found in embryonic fibroblasts (9), TC-PTP may also play an important role in the regulation of DNA synthesis of T and B cells.

In conclusion, we demonstrate that TC-PTP is involved in the functional and/or developmental regulation of both lymphocytes

and macrophages, and highlight the important role that TC-PTP may play in controlling immune responses. The inhibition of TC-PTP activity in macrophages is associated with immunosuppressive properties and may therefore represent a novel approach to control lymphocyte proliferation associated with autoimmune diseases and transplant rejection.

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