The Journal of Immunology

Myeloid-Derived Suppressor Cells Accumulate in Kidney Allograft Tolerance and Specifically Suppress Effector T Cell Expansion

Anne-Sophie Dugast, Thomas Haudebourg, Flora Coulon, Michèle Heslan, Fabienne Haspot, Nicolas Poirier, Romain Vuillefroy de Silly, Claire Usal, Helga Smit, Bernard Martinet, Pamela Thebault, Karine Renaudin and Bernard Vanhove

J Immunol 2008; 180:7898-7906; ;
doi: 10.4049/jimmunol.180.12.7898
http://www.jimmunol.org/content/180/12/7898

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Myeloid-Derived Suppressor Cells Accumulate in Kidney Allograft Tolerance and Specifically Suppress Effector T Cell Expansion[1]

Anne-Sophie Dugast,* Thomas Haudebourg,* Flora Coulon,* Michèle Heslan,* Fabienne Haspot,* Nicolas Poirier,* Romain Vuillefroy de Silly,* Claire Usal,* Helga Smit,* Bernard Martinet,* Pamela Thebault,* Karine Renaudin,† and Bernard Vanhove[2]*

The immune tolerance to rat kidney allografts induced by a perioperative treatment with anti-CD28 Abs is associated with a severe unresponsiveness of peripheral blood cells to donor Ags. In this model, we identified an accumulation in the blood of CD3$^-$class II$^-$CD11b$^+$CD80/86$^+$ plastic-adherent cells that additionally expressed CD172a as well as other myeloid markers. These cells were able to inhibit proliferation, but not activation, of effector T cells and to induce apoptosis in a contact-dependent manner. Their suppressive action was found to be under the control of inducible NO synthase, an enzyme also up-regulated in tolerated allografts. Based on these features, these cells can be defined as myeloid-derived suppressor cells (MDSC). Interestingly, CD4$^+$CD25^{high}FoxP3$^+$ regulatory T cells were insensitive in vitro to MDSC-mediated suppression. Although the adoptive transfer of MDSC failed to induce kidney allograft tolerance in recently transplanted recipients, the maintenance of tolerance after administration of anti-CD28 Abs was found to be dependent on the action of inducible NO synthase. These results suggest that increased numbers of MDSC can inhibit alloreactive T cell proliferation in vivo and that these cells may participate in the NO-dependent maintenance phase of tolerance. *The Journal of Immunology,* **2008, 180: 7898–7906.**

Although transplant tolerance in rats has frequently been associated with the action of regulatory T cells (Tregs)[3] (1–3), in some cases Tregs are not instrumental. For example, administration of anti-CD28 Abs (4) or anti-donor class II Abs (5) results in tolerance to kidney allografts across a full MHC barrier with an immune suppression that is at least partially dependent on non-T cells. In most cases, non-T cell populations with potential regulatory activity in the transplant setting have been identified as regulatory dendritic cells producing IDO (6) and heme oxygenase-1 (HO-1) (7), alternatively activated macrophages creating a Th2 environment (8), or NKT cells (9). Another type of non-T regulatory cell, so-called myeloid suppressor cells, have been associated with impaired immune reactivity to Ag challenge (mainly tumors but also infections), chronic inflammation, or superantigen-induced tolerance of CD4$^+$ T lymphocytes (reviewed by Sefarini et al. (10)). Myeloid suppressor cells have also been found to contribute to the immunosuppression accompanying the lethal systemic graft-vs-host reaction in irradiated mice (11). These cells are a heterogeneous mixture of myeloid cells at different stages of differentiation, including precursors of granulocytes, macrophages, dendritic cells, or early myeloid progenitors. To avoid confusion between mesenchymal stem cells and the commonly used term "myeloid suppressor cells", Gabrilovich et al. proposed the term "myeloid-derived suppressor cells" (MDSC) (12). In mice, these cells share the common functional characteristics of being able to inhibit T cell responses by inducing the apoptosis of activated T cells via up-regulation of NO production (13). NO regulates T cell activation via reversible disruption of the Jak/STAT5 signaling pathway (14). Additionally, arginase 1 can be induced in MDSC under the action of Th2-type cytokines, which synergize with NO to give rise to peroxynitrites that drive the apoptosis of Ag-primed T lymphocytes by inhibiting protein tyrosine phosphorylation via nitration of tyrosine residues (15). It has also been suggested that MDSC suppressive activity is mediated by CD4$^+$CD25$^+$ regulatory T cells and requires an interaction between CD152 on Tregs with CD80 that is up-regulated on MDSC upon contact with activated T lymphocytes (16). Mouse MDSC are defined phenotypically by their expression of CD11b and Gr1 (Ly-6G). The human equivalents are less well defined; they have been described as granulocyte-macrophage-progenitor cells expressing the CD34 marker (17). The rat counterparts express the CD11b/c and HIS48 myeloid markers (18).

Having previously identified non-T cells with suppressive activity in a rat model of kidney transplant tolerance (4), the aim of the current study was to characterize the phenotype and mechanisms of action of these cells. We report herein on an accumulation of suppressor cells within the peripheral blood and grafts of tolerant kidney graft recipients. These cells are characterized by their expression of CD80/86, signal regulatory protein α (SIRPα,

*Institut National de la Santé et de la Recherche Médicale, U643, Centre Hospitalier Universitaire Nantes, Institut de Transplantation et de Recherche en Transplantation, Université de Nantes, Faculté de Médecine, Nantes; and †Service d'Anatomie Pathologique du Centre Hospitalier Universitaire de Nantes, Nantes, France

Received for publication November 13, 2007. Accepted for publication April 14, 2008.

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 work was supported in part by the Roche Organ Transplant Research Foundation Grant 466230972 (to B.V.) and by the Progreffe Fundation.

[2] Address correspondence and reprint requests to Dr. Bernard Vanhove, Institut de Transplantation et de Recherche en Transplantation, Institut National de la Santé et de la Recherche Médicale U643, Centre Hospitalier Universitaire Hôtel Dieu, 30 boulevard Jean Monnet, 44093 Nantes Cedex 1, France. E-mail address: Bernard.Vanhove@univ-nantes.fr

[3] Abbreviations used in this paper: Treg, regulatory T cell; HO-1, heme oxygenase-1; iNOS, inducible NO synthase; L-NMMA, N^G-monomethyl-L-arginine; MDSC, myeloid-derived suppressor cells; 1-MT, 1-methyl-D,L-tryptophan; SIRPα, signal regulatory protein α; SnPP, tin protoporphyrin.

CD172a), CD11a/b, and inducible NO synthase (iNOS) and their ability to suppress T cell proliferation in a contact-dependent and iNOS-dependent manner. Additionally, our data demonstrate that these MDSC selectively suppress activated effector T cells, whereas natural $CD4^+CD25^{high}$ Tregs are largely resistant to this effect.

Materials and Methods

Animals and transplantations

Eight- to 12-wk-old male Lewis.1W (LEW.1W, haplotype $RT1^u$) and Lewis.1A (LEW.1A, haplotype $RT1^a$) congeneic rats (Centre d'Elevage Janvier, Le Genest-Saint-Isle, France) differed in their entire MHC region. Heterotopic LEW.1W kidney transplantation was performed as previously described (19). The kidney (right side) of the recipient (LEW.1A) was replaced by a LEW.1W donor allograft, and a contralateral nephrectomy was performed 7 days later, after which the allograft was life-sustaining. Studies described herein have been performed in accordance with the institutional guidelines of the Institut National de la Santé et de la Recherche Médicale (INSERM).

Tolerance induction

The JJ319 (IgG1 anti-rat CD28) mouse hybridoma was a gift from Dr. Thomas Hunig (Wurzburg, Germany). The JJ319 mAb was purified from hybridoma supernatant and administered to LEW.1A allograft recipients by i.p. injection at 1 mg/day for 7 days starting on the day of transplantation. This Ab induces a transient down-modulation of CD28 expression in vivo, without depleting target cells (20). Without treatment, the grafts were rejected 11 days posttransplantation. Syngeneic transplants (LEW.1A to LEW.1A) served as controls.

Abs and reagents

Purified anti-B7-1 (clone 3H5) and anti-B7-2 (clone 24F) mouse hybridomas were a gift from Dr. H. Yagita (Juntendo University School of Medicine, Tokyo, Japan). FITC-conjugated Pan-T (CD6), anti-CD11a (WT.1), anti-CD11b (WT.5), anti-RT1A (MHC class I), anti-CD40, and purified anti-CD45R (clone HIS24) were purchased from BD Pharmingen. HIS48 Ab was from Serotec. FITC-conjugated anti-CD172a (SIRPα or OX41), anti-CD4 (W3/25), anti-MHC class I (OX18), anti-MHC class II (OX6), anti-CD8 (OX8), anti-CD25 (OX39), anti-CD103 (OX62), anti-CD90 (OX7), and anti-CD62L (OX85) were prepared in our laboratory from the corresponding hybridomas obtained from the European Cell Culture Collection (Salisbury, U.K.). Alexa Fluor 647-conjugated anti-NKRP-1 (3.2.3) was prepared in our laboratory using a conjugation kit (Invitrogen: Molecular Probes). PE-conjugated anti-mouse was purchased from Jackson ImmunoResearch Laboratories. The anti-TCRβ R7-3 Ab was stained with a Zenon Alexa Fluor 700 mouse IgG1 labeling kit from Molecular Probes according to the manufacturer's instructions. Rabbit anti-rat iNOS Ab was purchased from Abcam.

Cells and cell sorting

Spleen T cells were prepared by nylon wool adhesion followed by depletion of NK cells, B cells, and monocytes using specific mAbs (clones 3.2.3, HIS24, and OX42, respectively), followed by anti-mouse IgG-coated Dynabeads (Invitrogen). Blood sampling was performed in heparin tubes. Erythrocytes were removed by hypotonic lysis. MDSC were identified by staining 30 min at 4°C with anti-rat CD80/86 mAbs and PE-conjugated anti-mouse IgG secondary Ab (Jackson ImmunoResearch Laboratories). After saturation with an excess of mouse IgG, cells were stained with FITC-conjugated anti-rat CD6 Ab and Alexa 647-conjugated anti-rat NKRP-1 Ab. Cells were then filtered (60 μm) and sorted using an Aria flow cytometer (BD Biosciences). Cell purity after sorting was routinely >95%. Bone marrow cells were isolated from the femurs and tibias of tolerant LEW.1A rats.

Quantitative real-time PCR

Quantitative real-time PCR was performed in an Applied Biosystems GenAmp 7700 sequence detection system using SYBR Green PCR core reagents. The following oligonucleotides were used: rat iNOS: upper primer 5′-GACCAAACTGTGTGCCTGGA-3′ and lower primer 5′-TACTCTGAGGGCTGACACAAGG-3′; and rat hypoxanthine phosphoribosyltransferase (HPRT): upper primer 5′-CCTTGGTCAAGCAGTA CAGCC-3′ and lower primer 5′-TTCGCTGATGACACAAACATGA-3′. HPRT was used as an endogenous control gene to normalize varying starting amounts of RNA. Relative expression between a given sample and a control sample, used for all experiments, was calculated with the $2(-\Delta\Delta C_t)$ method. All samples were analyzed in duplicate. Expression of genes of interest was compared between tolerant animals and syngeneic controls.

Adoptive cell transfer

Cell transfer was performed by i.v. injection into nonirradiated recipients on the day of kidney transplantation, as previously described (5). Donor cells were unfractionated spleen cells or MDSC purified from blood and bone marrow. In other experiments, CFSE-labeled alloreactive T cells were transferred into irradiated recipients and proliferated in vivo in a graft-vs-host disease-like manner, as previously described (21). Briefly, 150×10^6 spleen cells from LEW.1A rats were injected i.v. into allogeneic LEW.1W recipients that received a sublethal (4.5 Gy) total body irradiation on day −1. Eight million MDSC from the blood of LEW.1W rats was coinjected. On day 2.5, spleens were harvested and the number of $CFSE^+$ cells was analyzed by flow cytometry.

Cell culture and proliferation assays

MLRs were performed as previously described (21). Spleen dendritic cells, used as APC and stimulators, were enriched from LEW.1W spleens by a 14.5% Nicodenz gradient as previously reported (22). APC (10^5) and responding cells (10^5) were cocultured for 5 days in 96-well round-bottom culture plates in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10% heat-inactivated FCS, 1% nonessential amino acids, 5 mM HEPES, 1 mM sodium pyruvate, and 1 μM 2-ME. Proliferation was measured by addition of 0.5 μCi [^{3}H]thymidine per well. For polyclonal stimulation, T cells were stimulated with 2.6 μg/ml anti-CD28 Abs in flat-bottom 96-well plates previously coated with anti-CD3 (0.5 μg/ml; 2 h at 37°C). For transwell assays, 3×10^5 cells were placed in the lower and/or upper chamber of a 24-well Costar Transwell plate (Fisher Scientific) and cultured for 3 days. Proliferation was measured as for MLRs. In suppression assays, N^G-monomethyl-L-arginine (L-NMMA, Sigma-Aldrich) was used at 5 mM, 1-methyl-D,L-tryptophan (1-MT; Sigma-Aldrich) was used at 200 μM, and tin protoporphyrin (SnPP; Frontier Scientific) at 50 μM.

Mixed leukocyte reactions

MLRs were performed as previously described (21), except that 5 mM L-NMMA (Sigma-Aldrich) was added to the medium.

Cytotoxicity assay

Cytotoxicity was assessed in a ^{51}Cr-release assay. Target cells (YAC-1 cells, European Cell Culture Collection) were labeled with ^{51}Cr for 60 min at 37°C in complete medium. Serial dilutions of effector cells in complete medium were mixed with 3000 ^{51}Cr-labeled target cells in triplicate in V-bottom 96-well plates and centrifuged for 1 min at $1500 \times g$. Plates were then incubated for 4 h at 37°C, 5% CO_2. The supernatants were harvested and ^{51}Cr release was determined using a scintillation counter (PerkinElmer). Specific cytotoxicity was calculated according to the following formula: (experimental release − spontaneous release) × 100/(maximum release − spontaneous release).

Cytokine assays

IFN-γ and IL-2 in the supernatants were measured by ELISA using kits from BD Biosciences and R&D Systems, respectively, according to the manufacturers' instructions.

Immunostaining

Analyses of cell cytospins were performed after acetone fixation and saturation (using PBS, 4% BSA, 10% goat serum) for 30 min at room temperature. Staining with iNOS Abs (Abcam) was performed overnight at 4°C. Cells were washed 3 times and incubated for 1 h at room temperature with FITC-conjugated anti-rabbit IgG diluted 1/100 and mounted with Dako medium (DakoCytomation). Graft samples were embedded in Tissue Tek OCT compound, snap-frozen in liquid nitrogen, cut into 5-μm sections, and fixed in acetone. Sections were permeabilized for 30 min at room temperature using 0.5% saponin, 4% BSA, 2% normal goat serum, 10% rat serum in PBS, and stained with the primary Ab rabbit anti-iNOS (Abcam), FITC-conjugated anti-HIS48 mAb, and purified CD11b/c (OX42) mAb overnight at 4°C. After washing, sections were incubated for 1.5 h with the secondary Ab Alexa 350-conjugated anti-rabbit (4 μg/ml) (Invitrogen) and Alexa 568-conjugated anti-mouse IgG2a (2 μg/ml; Chemicon International), mounted with Dako medium, and analyzed using a confocal-like immunofluorescence technique (Zeiss Axiovert 200M microscope with an

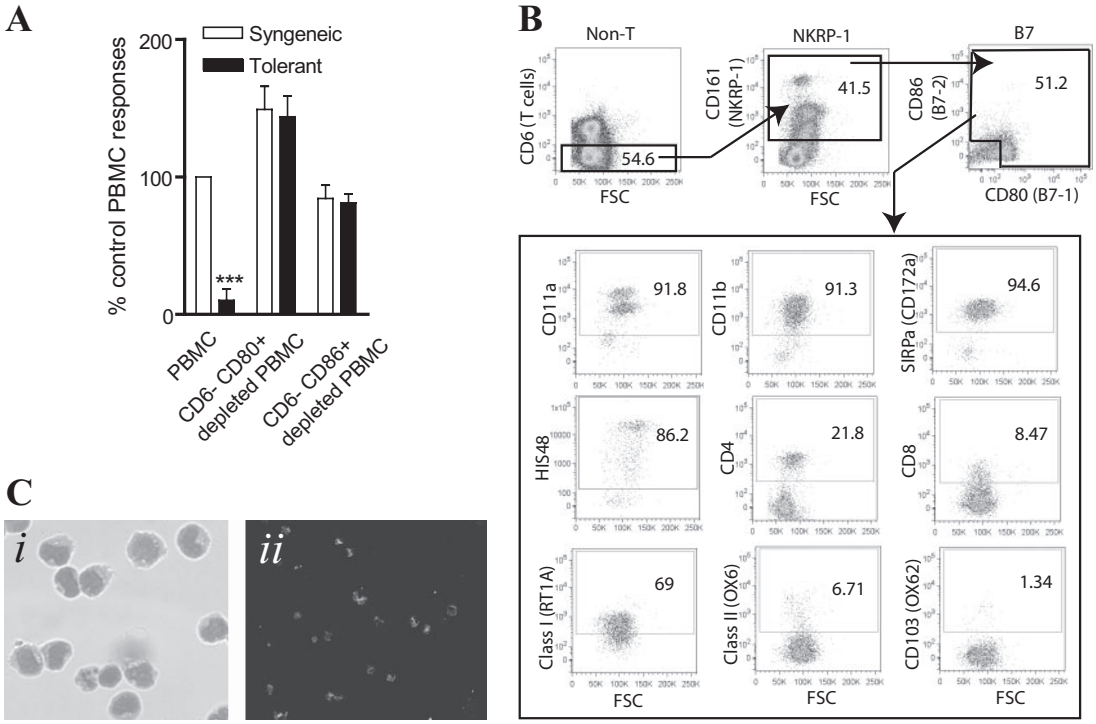

FIGURE 1. Characterization of non-T regulatory cells present in peripheral blood. *A*, Expression of CD80 and CD86. PBMC of the LEW.1A haplotype from syngeneic kidney graft recipients (open bars) or from tolerant kidney allograft recipients (3 mo posttransplantation; filled bars) were depleted of CD6 (pan-T marker)-negative/CD80$^+$ cells, CD6-negative/CD86$^+$ cells, or unmodified and stimulated with allogeneic LEW.1W APCs. [^{3}H]thymidine incorporation was measured after 5 days. *B*, Phenotype analysis. PBMC from tolerant kidney allograft recipients (3 mo posttransplantation) were analyzed by flow cytometry. Cells were gated on CD6$^-$NKRP-1$^+$CD80/86$^+$ cells and analyzed for the indicated markers. The percentages of cells expressing each marker are indicated. *Ci*, May-Grünwald Giemsa staining on cytospins of CD6$^-$NKRP-1$^+$CD80/86$^+$ cells sorted from PBMC from tolerant kidney allograft recipients (magnification ×63). *Cii*, Immunofluorescence analysis of iNOS expressed by CD6$^-$NKRP-1$^+$CD80/86$^+$ cells sorted from PBMC from tolerant kidney allograft recipients (magnification ×40).

Apotome module). Control sections were performed by replacing the primary Abs with dilution buffer. Naive kidneys were used as negative controls.

Western blot analysis

Cells extracts were prepared in RIPA buffer and quantified by BCA protein assay reagent (Pierce). Ten micrograms of total protein was resolved by 7.5% SDS-PAGE and transferred onto nitrocellulose membranes (ECL Hybond from GE Healthcare) using a Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad). Membranes were blocked with 5% skimmed milk for 2 h at room temperature and probed overnight at 4°C with 1 μg/ml anti-iNOS (Abcam) or 0.1 μg/ml β-actin (Santa Cruz Biotechnology) Abs and with secondary HRP-conjugated goat anti-rabbit Abs (Jackson ImmunoResearch Laboratories) or HRP-conjugated donkey anti-mouse, respectively, diluted 1/2000 in washing buffer. After washing, membranes were revealed using enhanced chemiluminesence (ECL Western blot, GE Healthcare) and exposed to Kodak X-Omat LS x-ray films.

FACS analyses

Cells were incubated with Alexa 647-conjugated 3.2.3 mAb, PE-conjugated CD80 mAb, and biotinylated-conjugated CD86 mAb for 20 min at 4°C. Cells were then washed twice and incubated with streptavidin-PECy7 for 20 min at 4°C. After 15 min saturation, cells were stained for 20 min with FITC-conjugated mAbs directed against rat CD Ags. To finish, cells were stained with Alexa 700-conjugated R7-3 or CD6 mAb for 30 min at room temperature. Analyses were performed using a FACScan II cytofluorometer (BD Biosciences) and FlowJo software. The negative gates were set using fluorescence-minus-one controls. Apoptosis was assessed by staining with APC-conjugated annexin V (BD Pharmingen) according to the manufacturer's instructions. Evaluation of T cell proliferation by flow cytometry was performed by staining of pure T cells with 5 μM CFSE (Molecular Probes) for 5 min at room temperature and measuring the FL-1 channel after cell culture.

Statistical analyses

Statistical significance was evaluated using a Mann-Whitney *U* test for the comparison of two groups. Graft survival was evaluated by Kaplan-Meier analysis using the log-rank test.

Results

Regulatory cell phenotype

We have previously demonstrated that administration of anti-CD28 Abs as an induction treatment results in immune tolerance to kidney allografts that is characterized by a hyporesponsiveness of recipient blood cells after challenge with donor alloantigens (4). In our earlier study, we identified a non-T cell population in the recipient blood that showed suppressive activity and was responsible for the inhibition of alloreactive T cells. These cells expressed NKRP-1, CD80, and/or CD86. Herein, we further characterize the phenotype of these cells. We first noticed that depletion of either CD80$^+$ or CD86$^+$ cells from the blood using magnetic beads totally restored the allogeneic proliferative response of PBMC against donor APC (Fig. 1*A*), suggesting that the regulatory cells in this model expressed CD80 and CD86. Next, we analyzed the phenotype of these cells after gating on non-T (CD6$^-$), CD80/86$^+$NKRP-1$^+$ cells and found them to express CD11a, CD11b, CD172a (SIRPα), and HIS48 (a rat marker usually associated with granulocytes (18)). Two subpopulations expressing high and intermediate levels of CD11a were observed. However, this variation in CD11a expression might have been induced by the isolation procedure (23). These cells additionally expressed class I (OX18) but not class II (OX6) or CD103 (OX62). Twenty-two percent of

these cells expressed CD4 and <10% expressed CD8 (Fig. 1*B*). None expressed CD40, CD90, CD25, CD62L, or HIS24 (data not shown). The absence of class II expression was confirmed by the fact that depletion of class II-positive cells using magnetic beads did not restore the alloreactivity of blood cells from the tolerant recipients (data not shown). After sorting by flow cytometry based on the $CD6^{-}CD80/86^{+}NKRP\text{-}1^{+}$ phenotype, the cells were found to adhere to plastic culture plates during overnight culture (data not shown). They presented a homogeneous myeloid-like morphology with a large, irregularly shaped nucleus and a large cytoplasm containing inclusions (Fig. 1*Ci*). These cells were also found to express iNOS (Fig. 1*Cii*).

Suppression assays

Because depletion of non-T $CD80/86^{+}$ cells from the blood of tolerant recipients restored the hyporesponsiveness of PBMC against alloantigens, we measured their suppressive activity after sorting. In mixed lymphocyte reactions these cells showed a robust dose-dependent suppressive activity on the proliferation of T cells stimulated by donor-derived APC (Fig. 2*A*, dotted bars). Moreover, these cells suppressed the proliferation of anti-CD3 + anti-CD28-stimulated T cells (Fig. 2*B*, open bars). As a control, non-T CD80/86-negative cells from the blood of the same animals displayed no suppressive activity (Fig. 2*B*, filled bars). The suppression was maximal 2 and 3 days after initiation of the culture and partial after 4 days (Fig. 2*C*). Cells with similar suppressive activity could also be isolated from the blood of control recipients of syngeneic grafts or of naive animals, although in the latter case fewer cells could be collected (see below for further details). The suppressive cells isolated from control-transplant recipients or from tolerant animals had the same suppressive activity on a per cell basis (Fig. 2*A*, striped and dotted bars, respectively). Cells with a phenotype comparable with the one observed in the blood (i.e., corresponding to the phenotype described in Fig. 1*B*) could also be detected in the spleen, lymph nodes, and bone marrow. These cells isolated from the bone marrow of tolerant recipients dose-dependently inhibited T cell activation down to a 1:10 ratio, as did similar cells from the blood. No such suppressive activity, however, could be measured after sorting from the spleen or lymph nodes (Fig. 2*D*).

Accumulation of MDSC in tolerant kidney graft recipients and function of MDSC in vivo

Given that MDSC from control and tolerant recipients had a similar suppressive activity on a per cell basis in the suppression assay (Fig. 2, *A* and *B*), we hypothesized that they might work in vivo as a result of their accumulation. We therefore compared the number of MDSC in the blood of tolerant allograft recipients to age-matched, syngeneic recipients and found a significant 2-fold accumulation in tolerant recipients (Fig. 2*E*). This increase in blood MDSC was not due to the tolerance induction regimen itself since the administration of anti-CD28 Abs to naive, nontransplant animals failed to result in their accumulation (data not shown).

To challenge the role of MDSC in vivo, we first tried to transfer tolerance with MDSC. However, the transfer of 2×10^6 MDSC isolated from the blood or the bone marrow did not significantly prolong kidney allograft survival (after the transfer of 2×10^6 MDSC in three recipients, kidney grafts survived 12, 14, and 16 days vs 11 days in untreated recipients). Transfers repeated on days 0, 3, and 6 were not more efficient. Additionally, the transfer of unsorted blood cells (25×10^6 cells) or spleen cells (200×10^6 cells) had no effect on allograft survival. However, using a previously described graft-vs-host disease-like system where CFSE-labeled T cells are infused into irradiated allogeneic recipients (21), we observed that coinjection of MDSC prevented the proliferation of allogeneic T cells in vivo (Fig. 2*F*).

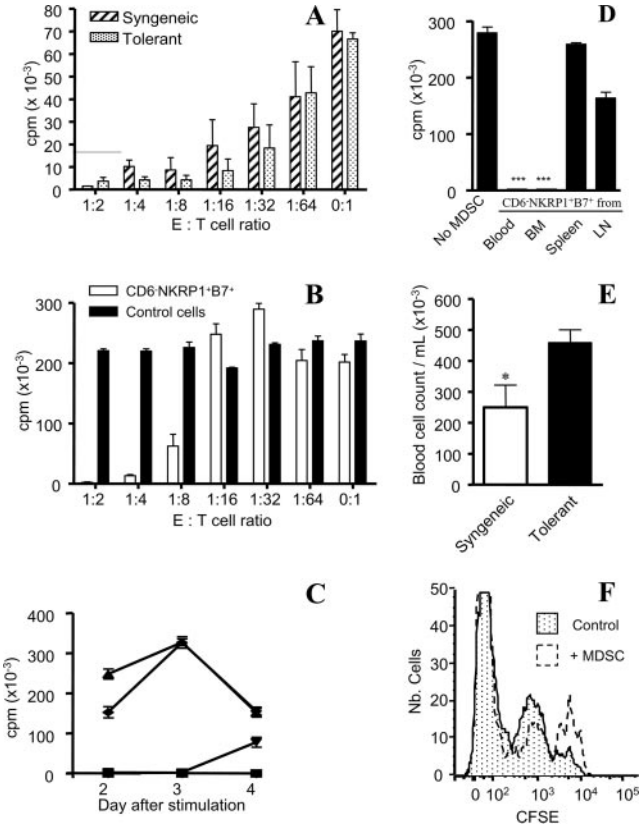

FIGURE 2. Suppressive activity of $CD6^{-}NKRP\text{-}1^{+}CD80/86^{+}$ MDSC. *A*, Suppression of mixed lymphocyte reactions. Purified LEW.1A T cells were stimulated with allogeneic LEW.1W APCs in the presence of the indicated ratios of effector ("E") $CD6^{-}NKRP\text{-}1^{+}CD80/86^{+}$ cells from the blood of tolerant allograft recipients or of recipients of syngeneic grafts. Shown are means ± SD of triplicate wells from one representative experiment out of six. *B*, Suppression of polyclonal stimulation. Purified LEW.1A T cells (10^5 cells/well) were stimulated with anti-CD3/CD28 in the presence of the indicated ratios of blood $CD6^{-}NKRP\text{-}1^{+}CD80/86^{+}$ cells or in the presence of control blood $CD6^{-}NKRP\text{-}1^{+}CD80/86^{-}$ cells from tolerant allograft recipients. [^{3}H]thymidine incorporation was measured after 3 days. Shown are means ± SD of triplicate wells from 1 representative experiment out of 10. *C*, Time-course of suppression. Same experiment as in *B*, performed at an E:T ratio of 1:4, where [^{3}H]thymidine incorporation was measured after 2, 3, and 4 days. *D*, Immune compartments containing suppressive cells. Purified T cells were stimulated with anti-CD3/CD28 mAbs in the presence of $CD6^{-}NKRP\text{-}1^{+}CD80/86^{+}$ cells (2.10^4) sorted from blood, bone marrow, spleen, and lymph nodes. [^{3}H]thymidine incorporation was measured after 3 days. *E*, Accumulation of MDSC in the blood of tolerant recipients. Blood samples collected on heparin were cleared of erythrocytes by hypotonic lysis and stained with CD6-FITC, NKR-P1 Alexa Fluor 647, CD80-PE, and CD86-PECy7. Total numbers of $CD6^{-}NKRP1^{+}CD80/86^{+}$ MDSC were evaluated in tolerant kidney allograft recipients (filled bar, $n = 5$) and in age-matched syngeneic kidney graft recipients (open bar, $n = 5$), 100 days posttransplantation. Results are expressed as mean cell number/ml blood ± SD. *, $p < 0.05$. Similar results were found in two other evaluations performed up to day 250 posttransplantation. *F*, In vivo inhibition of alloreactive T cell proliferation. CFSE-labeled spleen cells (150×10^6) were injected i.v. into irradiated allogeneic rat recipients. Half of the recipients additionally received 8×10^6 MDSC purified from blood. After 2.5 days, the relative abundance of $CFSE^{high}$ and $CFSE^{low}$ T cells was measured in the spleen by flow cytometry. Dotted histogram, controls; open histogram, coinjection of MDSC (one figure representative of two independent experiments).

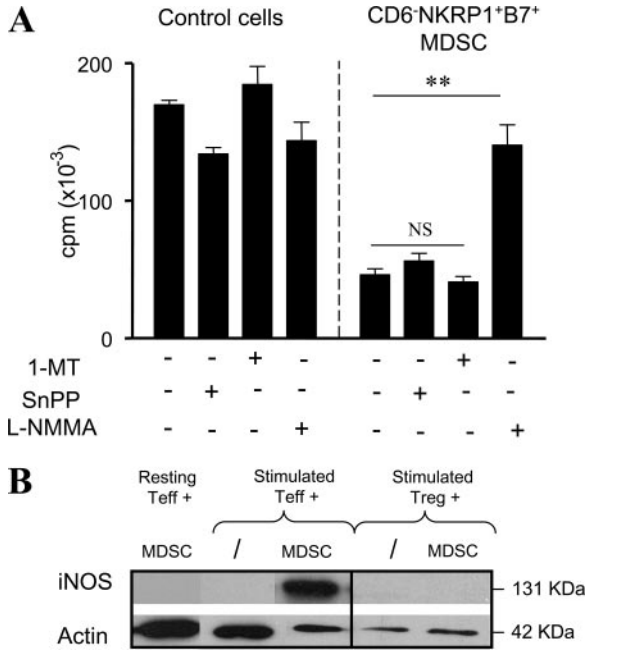

FIGURE 3. Mechanisms of action of $CD6^{-}NKRP\text{-}1^{+}CD80/86^{+}$ MDSC. *A*, Role of iNOS in the suppression. T cells from naive animals were stimulated with anti-CD3/CD28 Abs in the presence of a 1:3 E:T ratio of control $CD6^{-}NKRP\text{-}1^{+}CD80/86^{-}$ cells or $CD6^{-}$ $NKRP\text{-}1^{+}CD80/86^{+}$ MDSC extracted from the blood of tolerant kidney graft recipients, added to inhibit proliferation. Enzyme inhibitors were added to the cultures (L-NMMA, iNOS inhibitor; SnPP, HO-1 inhibitor; 1-MT, IDO inhibitor). Proliferation was measured after 3 days by [^{3}H]thymidine incorporation. Data are mean cpm ± SD of one representative experiment out of three. *B*, Western blot analysis of iNOS expression. Cells were cultured as in *A* for 2 days. Whole protein (10 μg) from the indicated cultures were resolved on a 7.5% SDS-PAGE containing 10 mM DTT and blotted onto nitrocellulose filters. Membranes were hybridized with anti-iNOS and actin Abs plus secondary Ab and revealed by chemiluminescence.

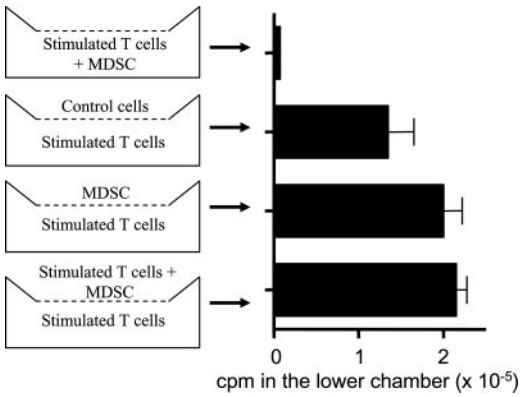

FIGURE 4. MDSC act in a contact-dependent manner. Transwell chambers were used to prevent direct cell contact between anti-CD3/CD28-stimulated T cells (6×10^5 cells from naive animals) and MDSC or control $CD6^{-}CD80/86^{-}$ cells (1.5×10^5 cells from the blood of tolerant kidney graft recipients). Proliferation was assessed in the lower chamber by [^{3}H]thymidine incorporation after 3 days of culture. Data are mean cpm ± SD of triplicate wells in two independent experiments.

Mechanisms of action

In the rat, high levels of NKRP-1 expression are characteristic of NK cells. Because NKRP-1 was expressed on non-T $CD80/86^{+}$ cells, we tested whether direct cytotoxicity could be responsible for their suppressive action. Using target YAC-1 cells, we observed an absence of cytotoxic activity, whereas control cells with the non-T CD80/86-negative $NKRP\text{-}1^{+}$ phenotype induced up to 60% cytotoxicity (data not shown), presumably because this cell population contained $NKRP\text{-}1^{high}$ NK cells. Therefore, direct NK-like cytotoxicity is not involved in the suppressive mechanism of the non-T $CD80/86^{+}NKRP\text{-}1^{+}$ cells. Because NO is known to be involved in several mechanisms of immunosuppression and because we detected iNOS expression by immunohistology (Fig. 1*Cii*), we next asked whether this enzyme also plays a role here. In suppression assays, a selective inhibitor of iNOS, L-NMMA (used at 5 mM), was able to reverse the suppression mediated by non-T $CD80/86^{+}NKRP\text{-}1^{+}$ cells (Fig. 3*A*). The finding that $CD11b/c^{+}$ cells suppress T cells via NO revealed that these cells had a phenotype and mechanism of action compatible with the definition of MDSC, as defined by Gabrilovitch et al. (12). Therefore, from this point on, the non-T $CD80/86^{+}NKRP\text{-}1^{+}$ cells are referred to as MDSC. We also noted that 1-methyl-DL-tryptophan (1-MT, used at 200 μM), an inhibitor of IDO, as well as tin protoporphyrin (SnPP, used at 50 μM), an inhibitor of HO-1, were both unable to block the suppressive activity of MDSC against stimulated T lymphocytes (Fig. 3*A*). Because iNOS was implicated in the suppression by MDSC, we further tested the level of iNOS expression by Western blot. iNOS was not expressed by freshly isolated blood MDSC, and resting and activated T cells expressed no or very little iNOS. Moreover, iNOS was not expressed when MDSC were mixed with resting T cells. In contrast, after contact between activated T cells and MDSC, iNOS was strongly up-regulated (Fig. 3*B*, *left section*). Similar experiments were performed using MDSC from the spleen and lymph nodes (cells that share a comparable phenotype were isolated from these compartments). In this case, however, no iNOS up-regulation was observed in any of the conditions tested (data not shown).

To determine whether cell contact between MDSC and target T cells was required for suppression, we performed transwell assays. Stimulated LEW.1A T cells were placed in the lower chamber with MDSC isolated from LEW.1A-tolerant kidney graft recipients in the upper chamber of the transwell. The physical separation abrogated the suppression, revealing a contact-dependent inhibition of the proliferative response (Fig. 4). Moreover, the physical separation from MDSC reduced the suppression even when the MDSC were mixed with other activated T cells, suggesting that activated T cells must be in contact with MDSC not only to elicit suppression but also to become sensitive to suppression (Fig. 4, *lower bar*). With the aim of identifying molecular interactions between MDSC and activated T cells that might be required for suppression, we tested several antagonistic Abs in the suppression assays. However, no modification of suppression could be obtained with Abs against CD80, CD86, CD80 + CD86 (tested at 10 μg/ml and at 50 μg/ml), class I, class II, CD11b/c, IFN-γ, CD172a, CD40, IL-4, or IL-10 (tested at 10 μg/ml; data not shown). To further understand how MDSC and NO blocked T cell proliferation, we analyzed their possible proapoptotic effect. Proliferation and apoptosis were measured using double staining with CFSE and annexin V after 2 days of culture. As shown in Fig. 5*Ai*, CFSE-labeled T cells proliferated after polyclonal stimulation and 40% of them were apoptotic. In the presence of MDSC, proliferation was minimal and 76.2% of the cells were found to be apoptotic. Thus, MDSC seem to affect the viability of stimulated T cells by blocking their proliferation and by inducing apoptosis in a contact-dependent manner. In similar assays, we also noticed that despite inhibiting T cell proliferation, MDSC only moderately prevented T cell activation after polyclonal stimulation, because ~60% of them

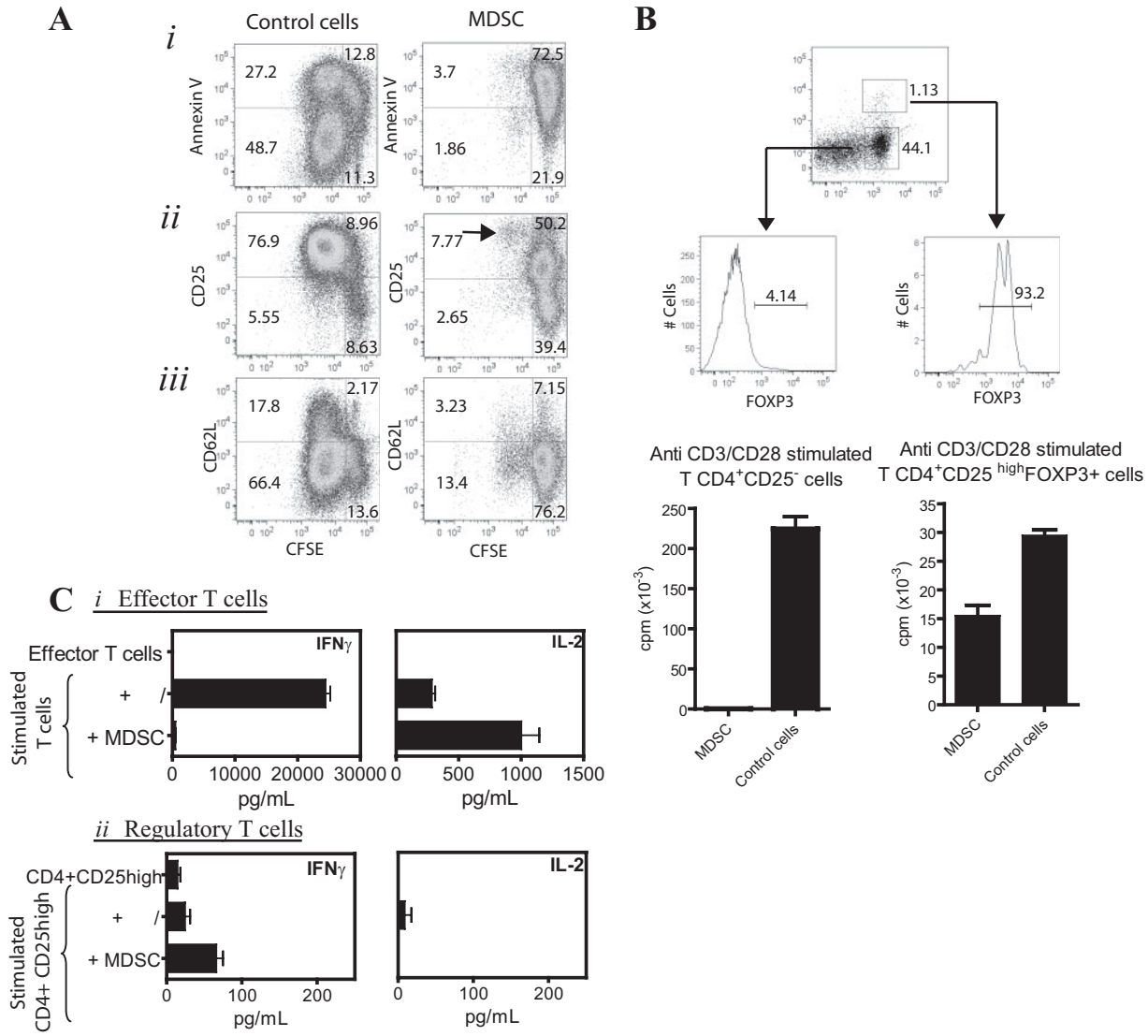

FIGURE 5. Effector function of MDSC. *A*, MDSC block proliferation but only moderately block activation of T lymphocytes. After coculture of anti-CD3/CD28-stimulated CFSE-labeled T cells with blood MDSC or control cells ($CD6^{-}CD80/86^{-}$) for 2 days, DAPI-negative cells were stained with annexin V (*i*), APC-conjugated CD25 (*ii*, arrow pointed on $CD25^{high}$ cells), or APC-conjugated CD62L (*iii*) and analyzed by flow cytometry. In these analyses, a threshold for CFSE fluorescence was set at a value of 100 to exclude CFSE-negative cells from the evaluation. *B*, Differential effect of MDSC on regulatory vs effector T cell proliferation. Spleen T cells were sorted into Tregs and effector T cells according to their $CD4^{+}CD25^{high}$ (these cells were mostly $FoxP3^{+}$) or $CD4^{+}CD25^{-}$ ($FoxP3^{-}$) phenotypes. Each population was stimulated with anti-CD3/CD28 and cultured with or without MDSC (ratio of 5 target cells for 1 MDSC) for 3 days. Proliferation was measured after 3 days by incorporation of $[^{3}H]$thymidine. Results are means ± SD of triplicate wells and representative of two experiments. *C*, Differential effect of MDSC on cytokine synthesis by regulatory vs effector T cells. Same experiment as in *B*, where supernatants were collected after 48 h. *i*, $CD4^{+}CD25^{-}$ effector T cells. *ii*, $CD4^{+}CD25^{high}$ regulatory T cells. IFN-γ and IL-2 production was measured by ELISA. Results are means of triplicate measurements ± SD from one experiment representative of four.

expressed CD25 and most had lost their expression of CD62L (Fig. 5, *Aii* and *Aiii*).

Differential effect on effector T cells and Tregs

According to the CFSE dilution assays after polyclonal activation, although MDSC blocked the proliferation of most $CD25^{+}$ T cells, a subpopulation of $CD25^{high}$ T cells escaped suppression (Fig. 5*Aii*, arrow), suggesting that $CD25^{+}$ Tregs might not be sensitive to MDSC. To directly measure this effect, we sorted $CD4^{+}CD25^{-}$ effector and $CD4^{+}CD25^{high}$ regulatory cells (93% of the $CD4^{+}CD25^{high}$ cells expressed FoxP3 in this assay) and tested the action of MDSC on the proliferation of these cell subpopulations. Effector T cells proliferated strongly after 3 days, and their proliferation could be fully inhibited by MDSC. In contrast, MDSC inhibited the proliferation of $CD4^{+}CD25^{high}FoxP3^{+}$ Tregs by 50% only (Fig. 5*B*). The cytokines secreted by these two T cell populations were also differentially affected by MDSC: stimulated $CD4^{+}CD25^{-}$ effector T cells produced less IFN-γ in the presence of MDSC, an observation compatible with the inhibition of their proliferation. In contrast, IL-2 production by stimulated effector T cells was not abolished by MDSC but rather was enhanced (Fig. 5*Ci*). Again, this is compatible with the idea that T cell proliferation, but not activation, is blocked by MDSC (IL-2 would therefore be released and, since not consumed by T cells, accumulate in the medium). As previously described (24), stimulated $CD4^{+}CD25^{high}FoxP3^{+}$ Tregs produced very low quantities of IL-2 and produced some IFN-γ that was enhanced by the addition of MDSC (Fig. 5C*ii*).

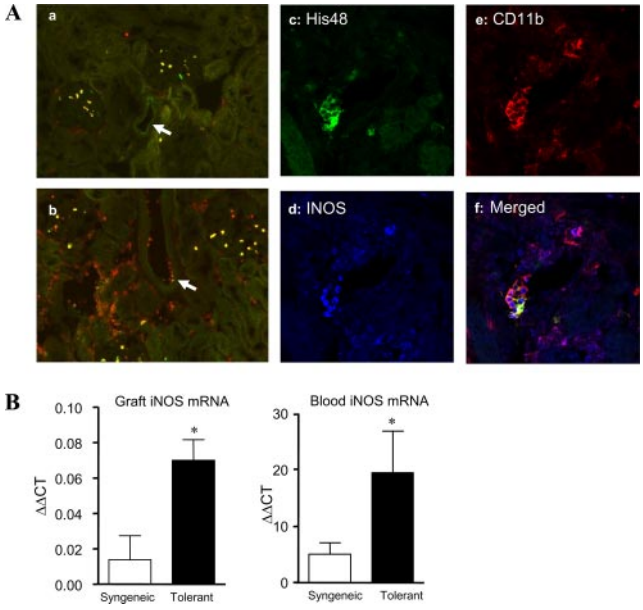

FIGURE 6. Graft infiltration and expression of iNOS. *A*, Immunohistological analysis of kidney grafts. Syngeneic grafts (*a*) and tolerated allografts (*b*) stained with CD11b (red fluorescence) and HIS48 (green fluorescence) Abs. Magnification ×10. The arrows indicate blood vessel sections. *c–f*, tolerated kidney allograft, at a magnification of ×40, focused on a blood vessel section, stained for HIS48 (*c*, green fluorescence), iNOS (*d*, blue fluorescence), CD11b (*e*, red fluorescence), and merged staining (*f*). *B*, Assessment of iNOS mRNA. The level of iNOS mRNA was analyzed by quantitative PCR in kidney grafts and blood from syngeneic or tolerant recipients 100 days after transplantation. *, $p < 0.05$; **, $p < 0.01$.

Graft infiltration and expression of iNOS

By immunohistology, we found $CD11b^+$ cells in the glomeruli of control and tolerated grafts (Fig. 6, *Aa* and *Ab*). In the parenchyma, $CD11b^+$ cells (Fig. 6*Ab*) synthesizing iNOS were found only in tolerant allografts (Fig. 6*Ae–f*). At higher magnification, $CD11b^+HIS48^+iNOS^+$ cells could be detected in the parenchyma and associated with the blood vessel walls (Fig. 6, *Ab* and *Ac–f*). Moreover, a quantitative analysis of messenger RNA for iNOS revealed a significantly higher expression in tolerated grafts than in syngeneic grafts (Fig. 6*B*). Also, more iNOS mRNA was visible in the blood of tolerant recipients (Fig. 6*B*).

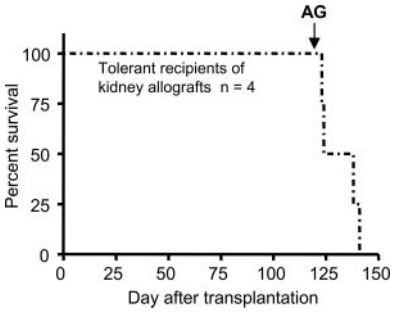

FIGURE 7. iNOS inhibition in vivo brakes tolerance. Four tolerant kidney allograft recipients (>120 days posttransplantation) received i.p. injections of 30 mg/kg of the iNOS inhibitor aminoguanidine (AG) every 12 h. Recipient survival is represented. Control rats also received aminoguanidine, and kidney function was recorded twice a week for 20 days. In these controls, kidney function was unmodified (uremia of 4 mmol/L and creatininemia of 18 μmol/L).

Role of MDSC in transplant tolerance

Because the tolerated kidney grafts were infiltrated with CD11b cells expressing iNOS (Fig. 6*A*) and accumulated more iNOS mRNA (Fig. 6*B*), it is likely that in tolerant recipients, MDSC accumulate and localize in the graft. To challenge the hypothesis that tolerance was achieved as a result of the activity of the iNOS enzyme, we tested the effect of injection of aminoguanidine, an inhibitor of iNOS, on tolerance 120 days after kidney transplantation. The results showed that tolerant recipients rejected their graft within an average of 10 days after aminoguanidine injection (100 mg/kg i.p. twice daily; Fig. 7). A pathological examination of these grafts revealed that acute, cellular-mediated rejection was the origin of the graft failure. These data demonstrate that the maintenance phase of tolerance in this model requires an active synthesis of NO.

Discussion

Herein, we show that the rat model of anti-CD28 Ab-induced kidney allograft tolerance triggers the accumulation of plastic-adherent $CD11b^+$ myeloid cells expressing CD80/86 that can be defined as MDSC. In vitro, these cells induced a contact-dependent apoptosis of activated effector T cells that themselves triggered the expression of iNOS by MDSC. MDSC had a limited effect on the proliferation of $CD4^+CD25^{high}FoxP3^+$ Tregs that failed to induce iNOS in MDSC. The action of NO production was critical to the immunosuppression mediated by MDSC and in maintaining the tolerant state in vivo.

It has become clear that transplant tolerance uses multiple cellular mechanisms that cooperate to suppress immunity, involving several types of regulatory T cells and tolerogenic DCs. Cooperation between different cell types might even be required to establish infectious tolerance to kidney allografts (5). In these situations, it is thought that CD152 up-regulated on Tregs interacts with CD80 on tolerogenic DCs in an Ag-cognate manner. On the one hand, this interaction results in the maintenance of Treg suppressive activity and, on the other hand, promotes a CD80-dependent (and IFN-γ-dependent) up-regulation of IDO, an enzyme that degrades the essential amino acid tryptophan (6). Tryptophan metabolites then suppress T cell responses as well as T cell clonal expansion. In vivo, CTLA-4/CD80 (and/or CD86) interactions have been shown to be required for tolerance after heart allotransplantation in mice (25), as well as IDO activity after heart allotransplantation in the rat (3). Tumors can also modulate immune responses by triggering an immune tolerance. In the latter case, although IDO induced by the action of local Tregs contributes to tumor-induced tolerance (26), the accumulation of MDSC appears to be a dominant mechanism in rodents as well as in man (10). The mechanism of action of MDSC typically involves the synthesis of NO (13) and/or the action of arginase 1 (27). Additionally, previous studies have suggested that mice $Gr\text{-}1^+CD11b^+$ MDSC express CD80 and suppress immune responses to tumors by promoting IDO up-regulation after engagement of CTLA-4 expressed by infiltrating activated or regulatory T cells (16). Herein, we show that the MDSC that accumulate in kidney allograft tolerance also express CD80. However, in our hands, CD80-CD152 interactions were not essential factors for MDSC function in vitro, because anti-CD80 Abs had no effect on the system. Nevertheless, the removal of $CD80^+$ cells as well as $CD86^+$ cells from the MLRs restored proliferation, suggesting that MDSC do express both markers. In addition, MDSC-mediated suppression in vitro was IDO independent, because it was not reversed by the IDO inhibitor 1-MT. As a comparison, 1-MT was shown to reverse the suppression driven by tolerogenic DC and by $CD11b^-$ monocytes

(4), which are different from the MDSC described here, in the same rat strain combination (5). Instead, in vitro, we found that the immunosuppressive activity of rat MDSC was solely controlled by NO. The fact that iNOS was detectable in isolated MDSC as well as in graft-infiltrating cells reinforces this idea. In vivo, the fact that injection of the iNOS inhibitor aminoguanidine induced the rejection of otherwise tolerated allografts also showed that the maintenance of the tolerant state was under the control of NO and not of IDO. In other tolerance models with the same rat strain combination, however, inducing rejection of tolerated allografts necessitated the administration of both IDO and iNOS inhibitors (28), indicating the possible coexistence of two mechanisms that cooperate to maintain transplant tolerance. Therefore, our data point toward a functional difference between mouse Gr-1^+CD11b$^+$ MDSC that mediate suppression via NO and possibly IDO and the rat MDSC described herein that appear not to use the IDO pathway.

In mice, MDSC function is also dependent on IFN-γ (29). In our rat system in vitro, we did not find a critical role for this factor, because anti-IFN-γ Abs failed to modify the suppression of MDSC on anti-CD3 + anti-CD28-activated T lymphocytes. Because SIRPα is an inhibitory receptor that modulates macrophage and DC function (30) and because it was expressed by rat MDSC, it was possible that SIRPα-CD47 interactions reinforced the suppressive activity of MDSC. However, the suppression by MDSC was not reduced by anti-SIRPα Abs either. Preventing CD40-CD40L molecular interactions or the action of IL-4 and IL-10 cytokines was also inefficient. Thus, the interactions required for MDSC function in the rat require further exploration.

Our investigations suggest that the MDSC-mediated suppression lacks Ag specificity, because MDSC could regulate the proliferation of third-party APC-stimulated T cells as well as the proliferation of anti-CD3 + anti-CD28-stimulated T cells. Additionally, MHC class II expression was not detected by flow cytometry and depleting MHC class II$^+$ cells from the blood did not prevent T cell unresponsiveness in the MLRs. Thus, it appears that MDSC do not interact with CD4$^+$ T cells in a cognate manner. Therefore, the capacity of MDSC to generate suppressive signals when encountering activated T cells most likely serves to regulate immune responses during times of heightened immune activity, without Ag specificity. In transplantation, MDSC might have a regulatory function, in cooperation with other, Ag-specific, regulatory cells. Herein, the observation that inhibition of iNOS in vivo induced rejection suggests that NO-based suppression mechanisms are not dispensable.

An important issue was the location in vivo where a contact could occur between effector T cells and blood MDSC. We detected MDSC in the blood of tolerant recipients of kidney allografts. In the spleen and lymph nodes, cells were identified that had a comparable phenotype but were devoid of ex vivo suppressive activity. By immunohistology, infiltrating cells expressing CD11b, HIS48, and iNOS markers could be detected in tolerated allografts, in the parenchyma, as well as being associated with blood vessel walls (CD80 and CD86 could not be detected by immunohistology in the rat species because the Abs do not bind to fixed tissues). Although we were unable to prove that these cells have a suppressive activity in situ, this phenotype is compatible with the presence of MDSC within the graft. Additionally, messenger RNA for iNOS was found to be accumulated 4-fold in tolerated kidneys. It is therefore possible that blood MDSC suppress T cells inside the graft. This mechanism of action would then be similar to tumor-infiltrating MDSC. In this kidney transplant model, however, tolerance is clearly associated with the control, and not the elimination, of alloreactive T cells, because they can be detected in the periphery several months after transplantation. Indeed, the simple removal of MDSC in vitro from blood cells collected on day 100 was sufficient to lift the suppression of lymphocyte alloreactivity, indicating that the Ag-specific lymphocytes had not been deleted in tolerant recipients. We have previously reported that donor-specific alloreactive T lymphocytes in the blood of tolerant recipients of kidney allografts expressed high rates of activation and apoptotic markers (4). This suggested that alloreactive T cells were continuously produced, presumably as a result of a thymic output, and kept under control by contact with MDSC, which as a result induced apoptosis not only in the graft but also in the blood. Herein, we confirmed in vitro that reactive T cells express activation markers (express CD25 and lose CD62L) and undergo apoptosis at a high rate in the presence of MDSC.

The question remains as to what extent MDSC participate to tolerance induction or maintenance in vivo. Although the direct evaluation of their role would be provided by an adoptive transfer of MDSC, correlated with tolerance, none of our trials including transfer of spleen cells, blood cells, blood MDSC, and bone marrow MDSC could induce tolerance. Instead, we found a slight, nonsignificant delay in the occurrence of rejection after transfer of blood or blood-derived MDSC. One reason might be that MDSC lose their suppressive activity after transfer, possibly as a result of differentiation. Another might be that NO could play a role in the maintenance phase but not in the induction phase of the tolerance that might be under the control of other mechanisms such as IDO, as previously shown in transplantation trials in the same rat strain combination (3, 28).

A challenging observation was that MDSC were also present in recipients of isografts as well as in naive animals. MDSC from control animals appeared to have a similar activity to those from tolerant recipients on a per cell basis. Therefore, the only difference was the increase in numbers of these cells in tolerant recipients. These features characterize MDSC as natural modulators of immune reactivity, mobilized by tumors, but also by tolerated allografts, to establish or reinforce tolerance. This view is strengthened by the recent observation that genetic inactivation of CD11b abolishes oral tolerance without compromising APC maturation or Ag-specific immune activation (31), establishing a specific role of CD11b$^+$ cells in oral tolerance induction.

A novel finding was that MDSC do express iNOS upon contact with activated T cells, but not upon contact with activated Tregs. Moreover, MDSC block the expansion of effector T cells and, to a lesser extent, CD4$^+$CD25$^+$FoxP3$^+$ Tregs. The fact that MDSC spare Tregs directly reinforces the suggestion by Yang et al. (16) that MDSC might mediate suppression at least in part via Tregs. However, in our study MDSC suppressed the proliferation of stimulated CD4$^+$CD25$^-$ T lymphocytes to the same extent as unsorted T cells, clearly showing that the presence of CD4$^+$CD25$^+$FoxP3$^+$ Tregs is not required, at least in vitro, for MDSC-mediated suppression.

In summary, our studies show a significant accumulation of MDSC in a rat model of kidney transplant tolerance. These cells have a nonspecific immunosuppressive activity in vivo and in vitro, involving the action of iNOS, which is up-regulated after contact with activated effector T cells but not with Tregs. These data illustrate a novel immunoregulatory mechanism associated with transplant tolerance.

Acknowledgments

We are grateful to S. Brouard, R. Josien, and J. Ashton-Chess for critically reading the manuscript, and we thank P. Hulin and the Institut National de la Santé et de la Recherche Médicale-IFR26 confocal microscopy platform.

Disclosures

The authors have no financial conflicts of interest.

References

1. Pirenne, J., H. Kitade, M. Kawai, T. Koshiba, B. Van Damme, C. Mathieu, and M. Waer. 2005. Regulatory cells, TH1/TH2 unbalance, and Ab-induced chronic rejection in operational tolerance induced by donor-specific blood transfusion. *Transplantation* 79: S25–S27.
2. Chiffoleau, E., G. Beriou, P. Dutartre, C. Usal, J. P. Soulillou, and M. C. Cuturi. 2002. Induction of donor-specific allograft tolerance by short-term treatment with LF15-0195 after transplantation: evidence for a direct effect on T-cell differentiation. *Am. J. Transplant.* 2: 745–757.
3. Guillonneau, C., M. Hill, F. X. Hubert, E. Chiffoleau, C. Herve, X. L. Li, M. Heslan, C. Usal, L. Tesson, S. Menoret, et al. 2007. CD40Ig treatment results in allograft acceptance mediated by $CD8^+CD45RC^{low}$ T cells, IFN-γ, and indoleamine 2,3-dioxygenase. *J. Clin. Invest.* 117: 1096–1106.
4. Haspot, F., C. Seveno, A. S. Dugast, F. Coulon, K. Renaudin, C. Usal, M. Hill, I. Anegon, M. Heslan, R. Josien, et al. 2005. Anti-CD28 antibody-induced kidney allograft tolerance related to tryptophan degradation and TCR class II B7 regulatory cells. *Am. J. Transplant.* 5: 2339–2348.
5. Degauque, N., D. Lair, A. Dupont, A. Moreau, G. Roussey, F. Moizant, F. X. Hubert, C. Louvet, M. Hill, F. Haspot, et al. 2006. Dominant tolerance to kidney allografts induced by anti-donor MHC class II antibodies: cooperation between T and non-T $CD103^+$ cells. *J. Immunol.* 176: 3915–3922.
6. Mellor, A. L., and D. H. Munn. 2004. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat. Rev. Immunol.* 4: 762–774.
7. Chauveau, C., S. Remy, P. J. Royer, M. Hill, S. Tanguy-Royer, F. X. Hubert, L. Tesson, R. Brion, G. Beriou, M. Gregoire, et al. 2005. Heme oxygenase-1 expression inhibits dendritic cell maturation and proinflammatory function but conserves IL-10 expression. *Blood* 106: 1694–1702.
8. Tzachanis, D., A. Berezovskaya, L. M. Nadler, and V. A. Boussiotis. 2002. Blockade of B7/CD28 in mixed lymphocyte reaction cultures results in the generation of alternatively activated macrophages, which suppress T-cell responses. *Blood* 99: 1465–1473.
9. Seino, K. I., K. Fukao, K. Muramoto, K. Yanagisawa, Y. Takada, S. Kakuta, Y. Iwakura, L. Van Kaer, K. Takeda, T. Nakayama, et al. 2001. Requirement for natural killer T (NKT) cells in the induction of allograft tolerance. *Proc. Natl. Acad. Sci. USA* 98: 2577–2581.
10. Serafini, P., C. De Santo, I. Marigo, S. Cingarlini, L. Dolcetti, G. Gallina, P. Zanovello, and V. Bronte. 2004. Derangement of immune responses by myeloid suppressor cells. *Cancer Immunol. Immunother.* 53: 64–72.
11. 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.
12. Gabrilovich, D. I., V. Bronte, S. H. Chen, M. P. Colombo, A. Ochoa, S. Ostrand-Rosenberg, and H. Schreiber. 2007. The terminology issue for myeloid-derived suppressor cells. *Cancer Res.* 67: 425–426.
13. Mazzoni, A., V. Bronte, A. Visintin, J. H. Spitzer, E. Apolloni, P. Serafini, P. Zanovello, and D. M. Segal. 2002. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *J. Immunol.* 168: 689–695.
14. Bingisser, R. M., P. A. Tilbrook, P. G. Holt, and U. R. Kees. 1998. Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. *J. Immunol.* 160: 5729–5734.
15. Brito, C., M. Naviliat, A. C. Tiscornia, F. Vuillier, G. Gualco, G. Dighiero, R. Radi, and A. M. Cayota. 1999. Peroxynitrite inhibits T lymphocyte activation and proliferation by promoting impairment of tyrosine phosphorylation and peroxynitrite-driven apoptotic death. *J. Immunol.* 162: 3356–3366.
16. Yang, R., Z. Cai, Y. Zhang, W. H. Yutzy, IV, K. F. Roby, and R. B. Roden. 2006. CD80 in immune suppression by mouse ovarian carcinoma-associated $Gr\text{-}1^+CD11b^+$ myeloid cells. *Cancer Res.* 66: 6807–6815.
17. Garrity, T., R. Pandit, M. A. Wright, J. Benefield, S. Keni, and M. R. Young. 1997. Increased presence of $CD34^+$ cells in the peripheral blood of head and neck cancer patients and their differentiation into dendritic cells. *Int. J. Cancer* 73: 663–669.
18. Prins, R. M., G. P. Scott, R. E. Merchant, and M. R. Graf. 2002. Irradiated tumor cell vaccine for treatment of an established glioma: II. Expansion of myeloid suppressor cells that promote tumor progression. *Cancer Immunol. Immunother.* 51: 190–199.
19. Soulillou, J. P., C. B. Carpenter, A. J. d'Apice, and T. B. Strom. 1976. The role of nonclassical Fc receptor-associated, Ag-B antigens (Ia) in rat allograft enhancement. *J. Exp. Med.* 143: 405–421.
20. Dengler, T. J., G. Szabo, B. Sido, W. Nottmeyer, R. Zimmerman, C. F. Vahl, T. Hunig, and S. C. Meuer. 1999. Prolonged allograft survival but no tolerance induction by modulating CD28 antibody JJ319 after high-responder rat heart transplantation. *Transplantation* 67: 392–398.
21. Haspot, F., F. Villemain, G. Laflamme, F. Coulon, D. Olive, J. Tiollier, J. P. Soulillou, and B. Vanhove. 2002. Differential effect of CD28 versus B7 blockade on direct pathway of allorecognition and self-restricted responses. *Blood* 99: 2228–2234.
22. Voisine, C., F. X. Hubert, B. Trinite, M. Heslan, and R. Josien. 2002. Two phenotypically distinct subsets of spleen dendritic cells in rats exhibit different cytokine production and T cell stimulatory activity. *J. Immunol.* 169: 2284–2291.
23. Stent, G., L. Irving, S. Lewin, and S. M. Crowe. 1995. The kinetics of surface expression of CD11/CD18 integrins and CD54 on monocytes and macrophages. *Clin. Exp. Immunol.* 100: 366–376.
24. Thornton, A. M., E. E. Donovan, C. A. Piccirillo, and E. M. Shevach. 2004. Cutting edge: IL-2 is critically required for the in vitro activation of $CD4^+CD25^+$ T cell suppressor function. *J. Immunol.* 172: 6519–6523.
25. Tsai, M. K., H. N. Ho, H. F. Chien, P. Ou-Yang, C. J. Lee, and P. H. Lee. 2004. The role of B7 ligands (CD80 and CD86) in CD152-mediated allograft tolerance: a crosscheck hypothesis. *Transplantation* 77: 48–54.
26. Munn, D. H., and A. L. Mellor. 2007. Indoleamine 2,3-dioxygenase and tumor-induced tolerance. *J. Clin. Invest.* 117: 1147–1154.
27. Bronte, V., P. Serafini, C. De Santo, I. Marigo, V. Tosello, A. Mazzoni, D. M. Segal, C. Staib, M. Lowel, G. Sutter, et al. 2003. IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. *J. Immunol.* 170: 270–278.
28. Guillot, C., S. Menoret, C. Guillonneau, C. Braudeau, M. G. Castro, P. Lowenstein, and I. Anegon. 2003. Active suppression of allogeneic proliferative responses by dendritic cells after induction of long-term allograft survival by CTLA4Ig. *Blood* 101: 3325–3333.
29. Kusmartsev, S. A., Y. Li, and S. H. Chen. 2000. $Gr\text{-}1^+$ myeloid cells derived from tumor-bearing mice inhibit primary T cell activation induced through CD3/CD28 costimulation. *J. Immunol.* 165: 779–785.
30. Latour, S., H. Tanaka, C. Demeure, V. Mateo, M. Rubio, E. J. Brown, C. Maliszewski, F. P. Lindberg, A. Oldenborg, A. Ullrich, et al. 2001. Bidirectional negative regulation of human T and dendritic cells by CD47 and its cognate receptor signal-regulator protein-α: down-regulation of IL-12 responsiveness and inhibition of dendritic cell activation. *J. Immunol.* 167: 2547–2554.
31. Ehirchiou, D., Y. Xiong, G. Xu, W. Chen, Y. Shi, and L. Zhang. 2007. CD11b facilitates the development of peripheral tolerance by suppressing Th17 differentiation. *J. Exp. Med.* 204: 1519–1524.
32. Taylor, S. R., D. R. Alexander, J. C. Cooper, C. F. Higgins, and J. I. Elliott. 2007. Regulatory T cells are resistant to apoptosis via TCR but not P2X7. *J. Immunol.* 178: 3474–3482.