JCI The Journal of Clinical Investigation

Monocytic suppressive cells mediate cardiovascular transplantation tolerance in mice

Mercedes Rodriguez Garcia, … , Jonathan S. Bromberg, Jordi C. Ochando

J Clin Invest. 2010;120(7):2486-2496. https://doi.org/10.1172/JCI41628.

Research Article **Transplantation**

One of the main unresolved questions in solid organ transplantation is how to establish indefinite graft survival that is free from long-term treatment with immunosuppressive drugs and chronic rejection (i.e., the establishment of tolerance). The failure to achieve this goal may be related to the difficulty in identifying the phenotype and function of the cell subsets that participate in the induction of tolerance. To address this issue, we investigated the suppressive roles of recipient myeloid cells that may be manipulated to induce tolerance to transplanted hearts in mice. Using depleting mAbs, clodronate-loaded liposomes, and transgenic mice specific for depletion of $CD11c^+$, $CD11b^+$, or $CD115^+$ cells, we identified a tolerogenic role for $CD11b^+CD115^+Gr1^+$ monocytes during the induction of tolerance by costimulatory blockade with CD40L-specific mAb. Early after transplantation, $Gr1^+$ monocytes migrated from the bone marrow into the transplanted organ, where they prevented the initiation of adaptive immune responses that lead to allograft rejection and participated in the development of Tregs. Our results suggest that mobilization of bone marrow $CD11b^+CD115^+Gr1^+$ monocytes under sterile inflammatory conditions mediates the induction of indefinite allograft survival. We propose that manipulating the common bone marrow monocyte progenitor could be a useful clinical therapeutic approach for inducing transplantation tolerance.

Monocytic suppressive cells mediate cardiovascular transplantation tolerance in mice

Mercedes Rodriguez Garcia,[1] Levi Ledgerwood,[2] Yu Yang,[3] Jiangnan Xu,[3] Girdhari Lal,[3] Bryna Burrell,[3] Ge Ma,[3] Daigo Hashimoto,[3] Yansui Li,[4] Peter Boros,[4] Marcos Grisotto,[5] Nico van Rooijen,[6] Rafael Matesanz,[7] Frank Tacke,[8] Florent Ginhoux,[9] Yaozhong Ding,[3] Shu-Hsia Chen,[3] Gwendalyn Randolph,[3] Miriam Merad,[3] Jonathan S. Bromberg,[3,4] and Jordi C. Ochando[1,3]

[1]Inmunología de Trasplantes, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain. [2]Department of Otolaryngology-Head and Neck Surgery, UC Davis Medical Center, Sacramento, California, USA. [3]Department of Gene and Cell Medicine and [4]Department of Surgery, Mount Sinai School of Medicine, New York, New York, USA. [5]Centro Universitário do Maranhão UNICEUMA, Sao Luis, Brazil. [6]Department of Molecular Cell Biology, VUMC, Amsterdam, The Netherlands. [7]Organización Nacional de Trasplantes, Madrid, Spain. [8]Medical Clinic III, University Hospital Aachen, Aachen University (RWTH), Aachen, Germany. [9]Singapore Immunology Network (SIgN), Agency for Science, Technology and Research (A*STAR), Singapore.

One of the main unresolved questions in solid organ transplantation is how to establish indefinite graft survival that is free from long-term treatment with immunosuppressive drugs and chronic rejection (i.e., the establishment of tolerance). The failure to achieve this goal may be related to the difficulty in identifying the phenotype and function of the cell subsets that participate in the induction of tolerance. To address this issue, we investigated the suppressive roles of recipient myeloid cells that may be manipulated to induce tolerance to transplanted hearts in mice. Using depleting mAbs, clodronate-loaded liposomes, and transgenic mice specific for depletion of $CD11c^+$, $CD11b^+$, or $CD115^+$ cells, we identified a tolerogenic role for $CD11b^+CD115^+Gr1^+$ monocytes during the induction of tolerance by costimulatory blockade with CD40L-specific mAb. Early after transplantation, $Gr1^+$ monocytes migrated from the bone marrow into the transplanted organ, where they prevented the initiation of adaptive immune responses that lead to allograft rejection and participated in the development of Tregs. Our results suggest that mobilization of bone marrow $CD11b^+CD115^+Gr1^+$ monocytes under sterile inflammatory conditions mediates the induction of indefinite allograft survival. We propose that manipulating the common bone marrow monocyte progenitor could be a useful clinical therapeutic approach for inducing transplantation tolerance.

Introduction

A major goal of clinical organ transplantation is to induce a donor-specific unresponsive state in a mature immune system that is free from long-term immunosuppression and chronic rejection. The general failure to reach this goal gives rise to 3 fundamental problems in clinical transplantation: (a) a high incidence of chronic rejection after the fifth year after transplant; (b) continuous need for immunosuppression with the risk of multiple side effects and opportunistic infections; and (c) discrepancy between the demand for and the availability of organs (1). To resolve these problems, there is a continuous search for novel therapeutic protocols to induce tolerance (2). Unfortunately, although experimental tolerogenic protocols have proved to induce indefinite allograft survival in mice or primates (3, 4), there are additional concerns that prevent translation of these methods into clinical practice (5) and underline the need for alternative tolerance-inducing protocols.

Here, we investigated the phenotype and function of various cell subsets of myeloid origin that are necessary for the induction of long-term allograft survival. One common approach to identifying the cells that exert a tolerogenic function is to specifically deplete cells in vivo and monitor the outcome of the immune response in the absence of the targeted cells. In experimental transplantation, the use of depletional mAbs and knockout or transgenic mouse strains has defined tolerogenic roles for Tregs (6), T cells (7), B cells (8), NK cells (9), and NKT cells (10). It is noteworthy that although much has been learned about the role of lymphocytes using depletional strategies, little is known about the outcome of allograft survival in the absence of cells of myeloid origin. Indirect evidence for the requirement for recipient myeloid cells during transplantation tolerance has been suggested. Auchincloss and colleagues reported that under costimulatory blockade, transplantation tolerance is not induced in recipients that do not express MHC class II in circulating leukocytes, consistent with the necessity of recipient MHC class II^+ myeloid cells for transplantation tolerance (11).

To investigate the requirement of myeloid cells for the induction of transplantation tolerance, vascularized BALB/c donor hearts were transplanted into fully allogeneic C57BL/6 recipients, and were treated with donor splenocyte transfusion (DST) plus anti-CD40L mAb for tolerance induction. Using recipient transgenic mice that express diphtheria toxin (DT) receptor (DTR) under the CD11c or CD11b promoter, together with depletional reagents against monocytes, macrophages, and neutrophils, we identified $CD11b^+CD115^+Gr1^+$ monocytes as suppressive cells that inhibit the immune response early after transplantation. Using adoptive

Authorship note: Mercedes Rodriguez Garcia and Levi Ledgerwood contributed equally to this work.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J Clin Invest.* 2010;120(7):2486–2496. doi:10.1172/JCI41628.

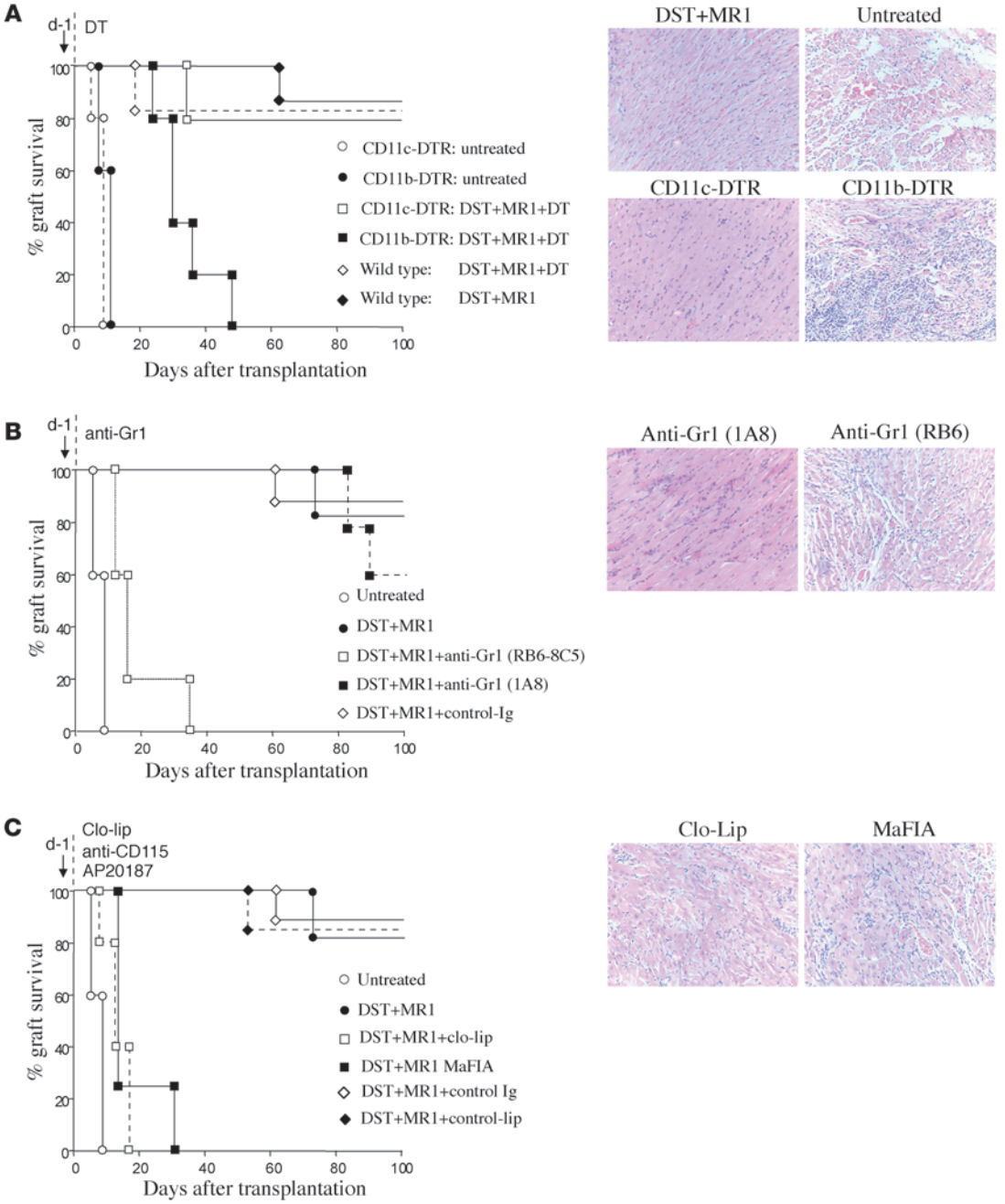

Figure 1

CD11b$^+$CD115$^+$Gr1$^+$ monocytes are required for tolerance induction to vascularized allografts. (**A**) Fully allogeneic vascularized cardiac grafts were accepted in tolerogen-treated (DST plus anti-CD40L mAb) CD11c-DTR mice (n = 10), but rejected in tolerogen-treated CD11b-DTR mice (n = 10), after DT administration. (**B**) Fully allogeneic vascularized cardiac grafts were rejected in tolerogen-treated mice that received the anti-Gr1 mAb RB6-8C5 (n = 10), but accepted in tolerogen-treated mice that received the anti-Gr1 mAb 1A8 (n = 10). Untreated rejecting controls were as in **A**. (**C**) Fully allogeneic vascularized cardiac grafts were rejected in tolerogen-treated mice that received clodronate loaded liposomes (clo-lip; n = 10) and in tolerogen-treated MaFIA mice (n = 10) following depletion of CD115-expressing cells. Untreated controls were as in **A**. Also shown are representative allograft images of H&E staining of the indicated groups at day of rejection or after 100 days of allograft survival. Original magnification, ×40.

transfer studies in recipients with reduced numbers of circulating CD11b$^+$CD115$^+$Gr1$^+$ monocytes, we further identified the anatomic locations and mechanisms of action by which these cells exert their immune regulatory function, which include antigen-nonspecific T cell suppression and development of Tregs. Finally, we provided evidence that manipulating the clonogenic bone marrow common macrophage/DC precursor (MDP) represents a promising therapeutic approach for the induction of indefinite allograft survival in solid organ transplantation, with concomitant therapeutic applications to clinical models of sterile inflammation.

Results

$CD11b^+CD115^+Gr1^+$ monocytes are necessary for tolerance induction. To identify the role of myeloid cells during the establishment of indefinite cardiac allograft survival, we targeted CD11c- and CD11b-expressing recipient cells, the major cell populations of myeloid origin. CD11c-DTR and CD11b-DTR mice express DTR under the control of the CD11c and CD11b promoters, and administration of DT in these mice depletes $CD11c^+$ DCs and $CD11b^+$ monocytes, macrophages, and neutrophils, respectively (12, 13). Vascularized BALB/c ($H\text{-}2^d$) hearts were transplanted into fully allogeneic C57BL/6 ($H\text{-}2^b$) CD11c-DTR or CD11b-DTR mice, and recipients were either left untreated for acute rejection or administered DST (1×10^7 cells i.v.) together with 500 μg anti-CD40L mAb on the day of transplantation for tolerance induction, as previously described (3). Figure 1A shows that transient depletion of CD11c-expressing cells prior to transplantation (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI41628DS1) did not interfere with the induction of transplantation tolerance in tolerogen-treated recipients. In contrast, when CD11b-expressing cells were depleted (Supplemental Figure 1), tolerance was not induced (Figure 1A), which suggested that CD11b-expressing cells were necessary for tolerance induction.

There are 2 main subsets of circulating monocytes that are characterized by their expression of CD11c and Gr1. $CD11c^+$ monocytes are $Gr1^-$, and $CD11c^-$ monocytes are $Gr1^+$ (14). Depletion of CD11c did not prevent tolerance induction (Figure 1A), which suggested that $CD11b^+Gr1^+$ monocytes might mediate tolerance induction. To investigate the tolerogenic role of $Gr1^+$ monocytes during tolerance induction, depletional doses of the anti-Gr1 mAb clone RB6-8C5 were administered to tolerogen-treated wild-type recipients. The results indicated that depletion of $Gr1^+$ cells prevented tolerance (Figure 1B and Supplemental Figure 1). Since anti-Gr1 mAb clone RB6-8C5 depletes both $Gr1^+$ monocytes and neutrophils under steady-state conditions (15), the tolerogenic role of $Gr1^+$ monocytes was further investigated by specifically depleting neutrophils with the anti-Gr1 mAb clone 1A8 (15). Consistent with previous results (16), depletion of neutrophils with 1A8 mAb did not interfere with tolerance induction (Figure 1B and Supplemental Figure 1), which suggested that blood circulating $Gr1^+$ monocytes mediated the induction of transplantation tolerance.

To confirm that monocytes were necessary for tolerance induction, circulating monocytes and macrophages were depleted with clodronate-loaded liposomes 24 hours before transplantation (Supplemental Figure 1 and ref. 17), and allograft survival was monitored. Depletion of monocytes with clodronate-loaded liposomes prevented tolerance induction (Figure 1C). The necessity of monocytes during tolerance induction was further investigated using transgenic MaFIA mice as recipients (18). MaFIA mice have an inducible Fas suicide/apoptotic system driven by the mouse CD115 promoter after exposure to the FK-binding protein dimerizer AP20187. CD115 is the M-CSF receptor (M-CSFR) and is involved in the survival and development of monocytes and macrophages (19). The results showed that depletion of monocytes in tolerogen-treated MaFIA recipients receiving AP20187 prevented the induction of transplantation tolerance (Figure 1C and Supplemental Figure 1).

Anatomic localization of tolerogenic $CD11b^+CD115^+Gr1^+$ monocytes. The anatomic location of $CD115^+CD11b^+Gr1^+$ monocytes and their trafficking requirements were next investigated. Monocytes are blood-circulating cells, and the results showed an increase of $CD115^+Gr1^+$ monocytes in the blood of transplanted recipients (Figure 2A). This suggested that $CD115^+Gr1^+$ monocytes were being generated in the bone marrow, which was confirmed by the observed increase of $CD115^+CD11b^+Gr1^+$ monocytes in the bone marrow of tolerized recipients (Figure 2B). To investigate the necessity of $Gr1^+$ monocyte mobilization from the bone marrow during tolerance induction, we used CCR2-deficient ($Ccr2^{-/-}$) mice, which have a 70% reduction in blood circulating $Gr1^+$ monocytes and simultaneous accumulation of these cells in the bone marrow caused by failure to exit the bone marrow (20). Analysis of blood circulating $CD115^+Gr1^+$ monocytes in tolerogen-treated $Ccr2^{-/-}$ recipient mice showed no increase in these cells (Supplemental Figure 2A), and tolerance could not be induced (Figure 2C), which suggested that bone marrow mobilization of $Gr1^+$ monocytes was necessary for tolerance induction. To investigate this hypothesis, $Gr1^+$ monocytes were mobilized in tolerogen-treated $Ccr2^{-/-}$ mice using a single dose of AMD3100, a selective antagonist of CXCR4 that induces rapid mobilization of bone marrow monocytes (Supplemental Figure 2A and refs. 21, 22). Figure 2C indicates that mobilization of bone marrow $Gr1^+$ monocytes restored indefinite allograft survival in tolerogen-treated $Ccr2^{-/-}$ recipients. Because treatment with AMD3100 mobilizes granulocytes in addition to $Gr1^+$ monocytes from the bone marrow, wild-type bone marrow $CD115^+CD11b^+Gr1^+$ monocytes (Supplemental Figure 2B) were adoptively transferred to $Ccr2^{-/-}$ recipients, and graft survival was monitored. Figure 2C indicates that transfer of wild-type bone marrow $CD115^+CD11b^+Gr1^+$ monocytes also induced tolerance in tolerogen-treated $Ccr2^{-/-}$ recipients. Together, these results showed that mobilization of $CD115^+CD11b^+Gr1^+$ from bone marrow to blood was necessary for tolerance induction.

Further analysis demonstrated an increase in $CD11b^+Gr1^+$ monocytes in the allografts of tolerogen-treated recipients (Figure 2D), suggestive of $Gr1^+$ monocyte trafficking from the blood to the transplanted organ during tolerance induction. To investigate the necessity of $CD115^+CD11b^+Gr1^+$ monocyte trafficking into the allograft, we manipulated P- and E-selectins, mediators of monocyte entry into inflamed tissues (23), by using BALB/c hearts from P- and E-selectin double-deficient ($PE^{-/-}$) donors. The results indicated that $PE^{-/-}$ allografts were rapidly rejected, accompanied by a significant decrease in $Gr1^+$ monocytes present in the allograft (Figure 2E and Supplemental Figure 2C). Blocking donor P- and E-selectin expression in the transplanted allograft prevented not only $CD11b^+Gr1^+$ monocytes, but also other cells, from trafficking into the allograft. To further investigate the unique role of monocytes during tolerance induction in the allograft, tolerogen-treated $Ccr2^{-/-}$ recipients were adoptively transferred with $CD115^+CD11b^+Gr1^+$ monocytes from fucosyltransferase IV-VII double-deficient ($FucTIV\text{-}VII^{-/-}$) C57BL/6 mice, and allograft survival was monitored. Circulating leukocytes express the ligands for P- and E-selectins (PSGL-1 and ESL-1) plus L-selectin, while endothelial cells express P- and E-selectins plus the ligands for L-selectin. Fucosyltransferase IV-VII is involved in the expression of selectin ligands. Therefore, monocytes from $FucTIV\text{-}VII^{-/-}$ mice are deficient in P- and E-selectin ligands and are prevented from tissue migration, while they are not deficient in L-selectin, which is necessary for lymph node homing (24). Adoptive transfer results indicated that unlike wild-type $CD11b^+Gr1^+$ monocytes, $FucTIV\text{-}VII^{-/-}$ $Gr1^+$ monocytes were unable to migrate into the transplanted organ (Supplemental Figure 2D) and did not induce indefinite allograft survival (Figure 2E). This suggested that $CD115^+CD11b^+Gr1^+$ monocyte trafficking into the grafted heart was necessary for tolerance induction.

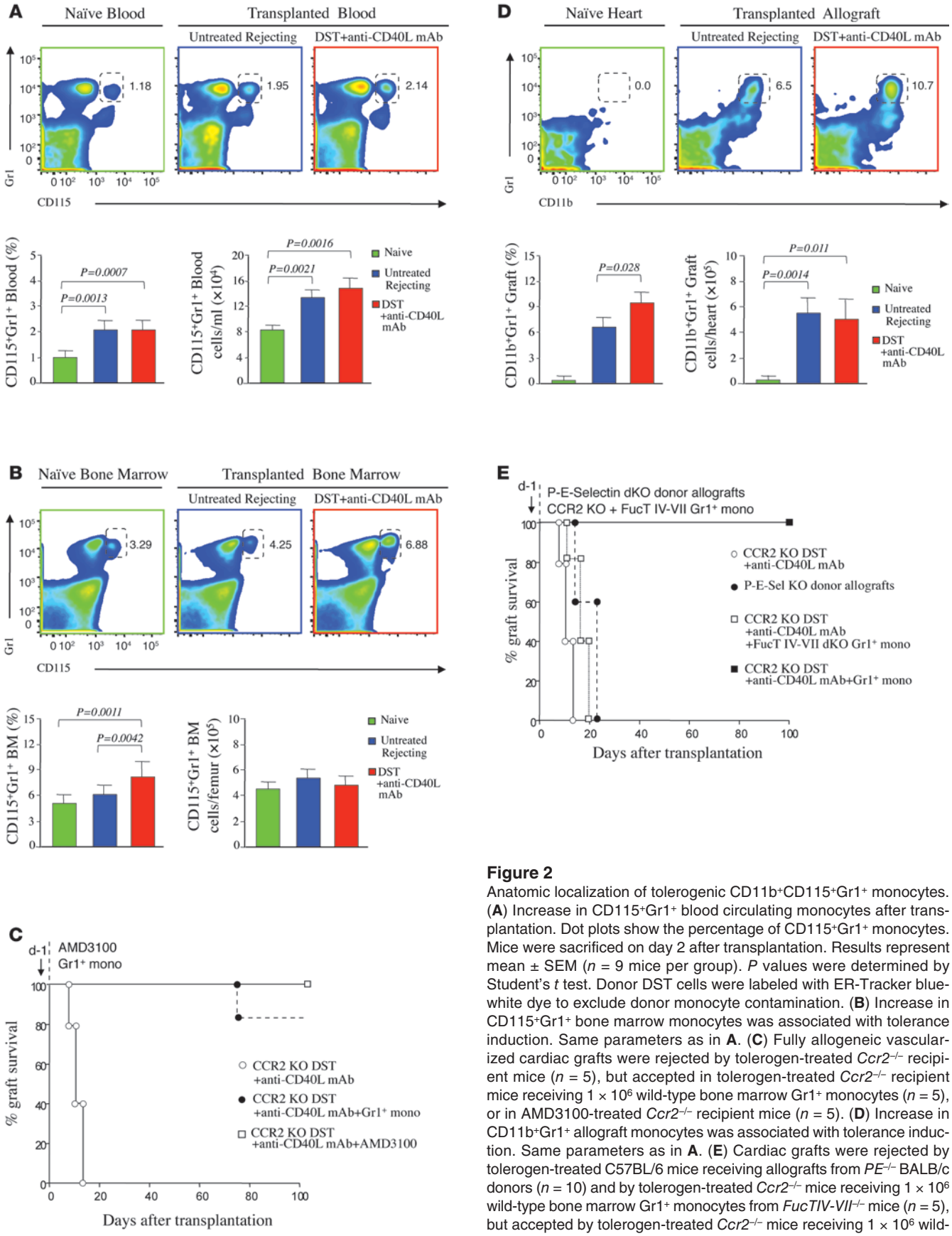

Figure 2

Anatomic localization of tolerogenic CD11b⁺CD115⁺Gr1⁺ monocytes. (**A**) Increase in CD115⁺Gr1⁺ blood circulating monocytes after transplantation. Dot plots show the percentage of CD115⁺Gr1⁺ monocytes. Mice were sacrificed on day 2 after transplantation. Results represent mean ± SEM (n = 9 mice per group). P values were determined by Student's t test. Donor DST cells were labeled with ER-Tracker blue-white dye to exclude donor monocyte contamination. (**B**) Increase in CD115⁺Gr1⁺ bone marrow monocytes was associated with tolerance induction. Same parameters as in **A**. (**C**) Fully allogeneic vascularized cardiac grafts were rejected by tolerogen-treated *Ccr2*$^{-/-}$ recipient mice (n = 5), but accepted in tolerogen-treated *Ccr2*$^{-/-}$ recipient mice receiving 1 × 10⁶ wild-type bone marrow Gr1⁺ monocytes (n = 5), or in AMD3100-treated *Ccr2*$^{-/-}$ recipient mice (n = 5). (**D**) Increase in CD11b⁺Gr1⁺ allograft monocytes was associated with tolerance induction. Same parameters as in **A**. (**E**) Cardiac grafts were rejected by tolerogen-treated C57BL/6 mice receiving allografts from *PE*$^{-/-}$ BALB/c donors (n = 10) and by tolerogen-treated *Ccr2*$^{-/-}$ mice receiving 1 × 10⁶ wild-type bone marrow Gr1⁺ monocytes from *FucTIV-VII*$^{-/-}$ mice (n = 5), but accepted by tolerogen-treated *Ccr2*$^{-/-}$ mice receiving 1 × 10⁶ wild-type bone marrow Gr1⁺ monocytes (n = 5).

Mechanisms of action of tolerogenic CD11b$^+$CD115$^+$Gr1$^+$ monocytes. Suppressive CD11b$^+$Gr1$^+$ monocytes have been shown in other model systems to mediate their inhibitory function through IFN-γ–dependent pathways (25, 26). To investigate the mechanisms by which CD11b$^+$Gr1$^+$ monocytes exert their tolerogenic function in transplantation, CD115$^+$CD11b$^+$Gr1$^+$ bone marrow monocytes from IFN-γ–deficient (*Ifng*$^{-/-}$) and IFN-γ receptor–deficient (*Ifngr*$^{-/-}$) mice were adoptively transferred into tolerogen-treated *Ccr2*$^{-/-}$ mice, and allograft survival was monitored. Tolerance was induced by adoptively transferred bone marrow CD11b$^+$Gr1$^+$ monocytes from *Ifng*$^{-/-}$ mice. In contrast, tolerance was not induced with adoptively transferred CD11b$^+$Gr1$^+$ bone marrow monocytes from *Ifngr*$^{-/-}$ mice (Figure 3A). These results suggested that CD11b$^+$Gr1$^+$ monocytes mediated their suppressive effect though IFN-γR signaling. NO, one of the downstream pathways for IFN-γR, is expressed in CD11b$^+$Gr1$^+$-suppressive cells (26), and Vanhove and colleagues have recently described the expression of iNOS in myeloid-derived suppressor cells (MDSCs) that mediate transplantation tolerance (27). We investigated the necessity of iNOS expression by CD115$^+$CD11b$^+$Gr1$^+$ monocytes during transplantation tolerance and found that tolerance was not induced in tolerogen-treated *Ccr2*$^{-/-}$ recipients that were transferred with iNOS-deficient (*Nos2*$^{-/-}$) bone marrow monocytes (Figure 3A). Moreover, monocytes deficient in molecules that participate in the signaling pathway between IFN-γR and iNOS (STAT-1 and IRF-1) were also unable to restore tolerance in tolerogen-treated *Ccr2*$^{-/-}$ recipients (Figure 3A). Immunohistochemistry showed that iNOS-expressing cells were present next to graft vascular endothelial cells in tolerogen-treated recipient allografts (Figure 3B) and that CFSE-labeled CD115$^+$CD11b$^+$Gr1$^+$ transferred monocytes expressed iNOS in tolerant allografts (Figure 3C). Others have previously shown that inflammatory monocytes require combined iNOS, arginase, and IL-4R expression to mediate their suppressive function (28, 29). CD11b$^+$Gr1$^+$ cells obtained from tolerized allografts upregulated mRNA for *iNOS* and *arginase*, but not *IL-4R* (Figure 3D), consistent with previous results (30). In mixed leukocyte reactions performed using allogeneic BALB/c (H-2^d) or third-party CBA (H-2^k) as stimulators and syngeneic C57BL/6 T cells as responders, sorted CD11b$^+$Gr1$^+$ monocytes from the allograft of tolerogen-treated recipients suppressed T cell proliferation in an antigen-nonspecific manner, whereas CD11b$^+$Gr1$^+$ monocytes from the bone marrow and the spleen of the same recipients did not suppress T cell proliferation (Figure 3E and data not shown). It has been suggested that CD115-expressing MDSCs mediate their suppressive function through the programmed cell death ligand 1 (PD-L1; ref. 31), which is necessary for transplantation tolerance (32, 33). Figure 3F indicated that CD11b$^+$Gr1$^+$ monocytes in tolerized allografts did not express PD-L1. Interestingly, the CD11b$^+$Gr1$^-$ subset did express high levels of PD-L1, which suggested that monocytes expressing PD-L1 could be involved in transplantation tolerance through the development of Tregs (34).

Tolerogenic monocytes are required for Treg development. Monocytic suppressor cells expressing CD115 have been recently shown to mediate Treg development in vivo (35). To monitor Treg development by monocytic suppressor cells in vivo, we crossed CD115-GFP MaFIA mice (H-2^b) with FoxP3-RFP mice (H-2^b) to obtain mice in which Tregs could be monitored following monocyte depletion. BALB/c (H-2^d) hearts were transplanted into fully allogeneic tolerogen-treated MaFIA/Foxp3 (H-2^b) recipients with or without 0.55 mg/ml AP20187 injected i.p. on days –5, –4, –3, –2, and –1, and allograft survival was monitored. Tolerance could not be induced in these recipients following monocyte depletion with AP20187 (data not shown). Treg development was investigated by examining Foxp3 expression in the blood and allograft of these recipients by flow cytometry; in AP20187-treated MaFIA/Foxp3 recipients, Tregs did not develop with time (Figure 4, A–C). To further demonstrate that CD115 monocytes mediate Treg development, 1×10^6 CD4$^+$Foxp3$^-$ T cells were sorted, labeled with CellVue-APC, and adoptively transferred into 3-week-old tolerogen-treated MaFIA/Foxp3 recipient mice with or without AP20187. CD4$^+$Foxp3$^+$ T cells could be isolated from the allografts of tolerogen-treated recipients 3 weeks after transfer (Figure 4D); however, monocyte depletion with AP20187 abrogated the ability of CD4$^+$Foxp3$^-$ T cells to become Foxp3$^+$. To further determine the role of CD115 monocytes in the development of Tregs, CD115-GFP cells from the allografts of tolerogen-treated MaFIA recipients with or without AP20187 were sorted 3 weeks after transplantation and cultured with CellVue-labeled CD4$^+$Foxp3$^-$ T cells for 72 hours, after which Foxp3 induction was monitored by flow cytometry. Only monocytes from the allografts of tolerant recipients were able to induce Foxp3 expression (Figure 4E).

Therapeutic manipulation of monocyte precursors. To design therapeutic protocols for the induction of transplantation tolerance, we used the MDP, which gives rise to monocytes, macrophages, conventional DCs, and plasmacytoid DCs; conversely, the common DC precursor (CDP) gives rise to DCs and plasmacytoid DCs, but not monocytes (36–38). MDP or CDP were adoptively transferred to tolerogen-treated *Ccr2*$^{-/-}$ recipients, and allograft survival was monitored. Adoptively transferred MDP, but not CDP, gave rise to blood circulating monocytes (Figure 5A). Tolerance was not induced in tolerogen-treated *Ccr2*$^{-/-}$ recipients that received CDP (Figure 5B). In contrast, tolerance was induced in *Ccr2*$^{-/-}$ recipients that received MDP, which suggested that manipulation of CD115-expressing monocyte precursors may represent a novel therapeutic approach for the induction of transplantation tolerance.

Discussion

We identified CD11b$^+$CD115$^+$Gr1$^+$ suppressive bone marrow monocytes as tolerance-inducing cells that accumulated in cardiac allografts early after transplantation and mediated the development of indefinite allograft survival. Identifying CD11b$^+$CD115$^+$Gr1$^+$ monocytes as suppressive cells that mediate transplantation tolerance contributes to the resolution of the longstanding quest to find a tolerogenic cell of myeloid origin. Past evidence suggested that a variety of MHC II$^+$ myeloid cells presumed to be DCs were required for tolerance (11), yet, despite an exhaustive search for a tolerogenic DC subpopulation in transplantation (39), accumulating evidence supports the idea that the same DC subset is implicated in both immunity and tolerance (40). However, a recently described myeloid-derived cell subpopulation (41) seems to favor tolerance via impairing T lymphocyte reactivity by inhibiting only activated T lymphocytes (42), while favoring the development of Tregs (35). These tolerogenic cells, the MDSCs, correspond phenotypically and functionally to the cells that we have characterized here. These CD11b$^+$CD115$^+$Gr1$^+$ bone marrow monocytes appear to be different from the novel monocyte reservoir population previously identified in the spleen (43), since tolerance could be induced in recipient mice following splenectomy (data not shown).

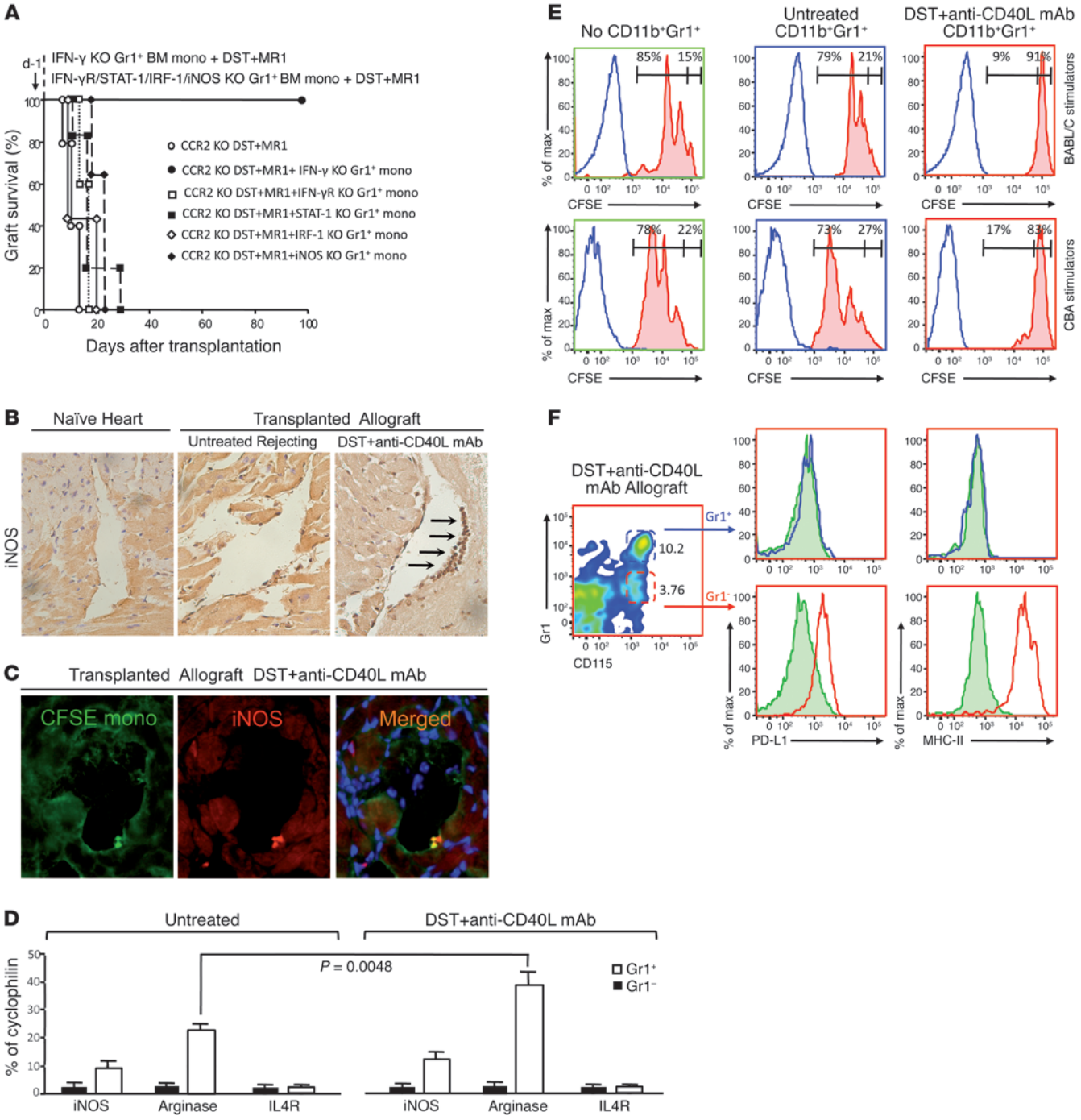

Figure 3

Mechanisms of action of tolerogenic CD11b$^+$CD115$^+$Gr1$^+$ monocytes. (**A**) Cardiac allografts were accepted by tolerogen-treated *Ccr2*$^{-/-}$ mice receiving 1 × 10^6 bone marrow Gr1$^+$ monocytes from *Ifng*$^{-/-}$ mice, but rejected by mice receiving 1 × 10^6 bone marrow Gr1$^+$ monocytes from *Ifngr*$^{-/-}$, *Stat1*$^{-/-}$, *Irf1*$^{-/-}$, or *Nos2*$^{-/-}$ mice (n = 5 per group). (**B**) Representative images of immunohistochemical analysis of iNOS$^+$ cells around graft endothelial cells 2 days after transplantation (n = 5 mice per group). Original magnification, ×40. (**C**) Fluorescent immunohistochemistry of CFSE-labeled adoptively transferred monocytes stained with iNOS in the allograft (n = 2 ± 0.3 cells/vessel; 20 vessels/heart; n = 3 mice). Original magnification, ×40. (**D**) Real-time RT-PCR of *iNOS*, *arginase*, and *IL-4R* expression in CD11b$^+$Gr1$^+$ monocytes in the allograft 2 days after transplantation. Data are representative of 3 independent experiments; SEM of PCR triplicates are shown. P values were determined by Student's t test. (**E**) Freshly isolated CD3$^+$CD4$^+$ T cells from C57BL/6 mice were sorted and labeled with CFSE, and 5 × 10^4 cells/well were cultured with CD11b$^+$Gr1$^+$ allograft monocytes (2.5 × 10^4 cells/well) in the presence of BALB/c or CBA APC (2.5 × 10^4 cells/well) for 72 hours. Proliferation of T cells was measured by CFSE dilution. Percentages of divided and undivided cells are shown. Data are representative of 3 independent experiments. (**F**) Expression of PD-L1 and MHC-II in CD115$^+$Gr1$^+$ and CD115$^+$Gr1$^-$ monocytes in the allografts of tolerant mice. Mice were sacrificed on day 2 after transplantation. Results are mean ± SEM (n = 5 mice). Donor DST cells were labeled with ER-Tracker blue-white dye to exclude donor monocyte contamination.

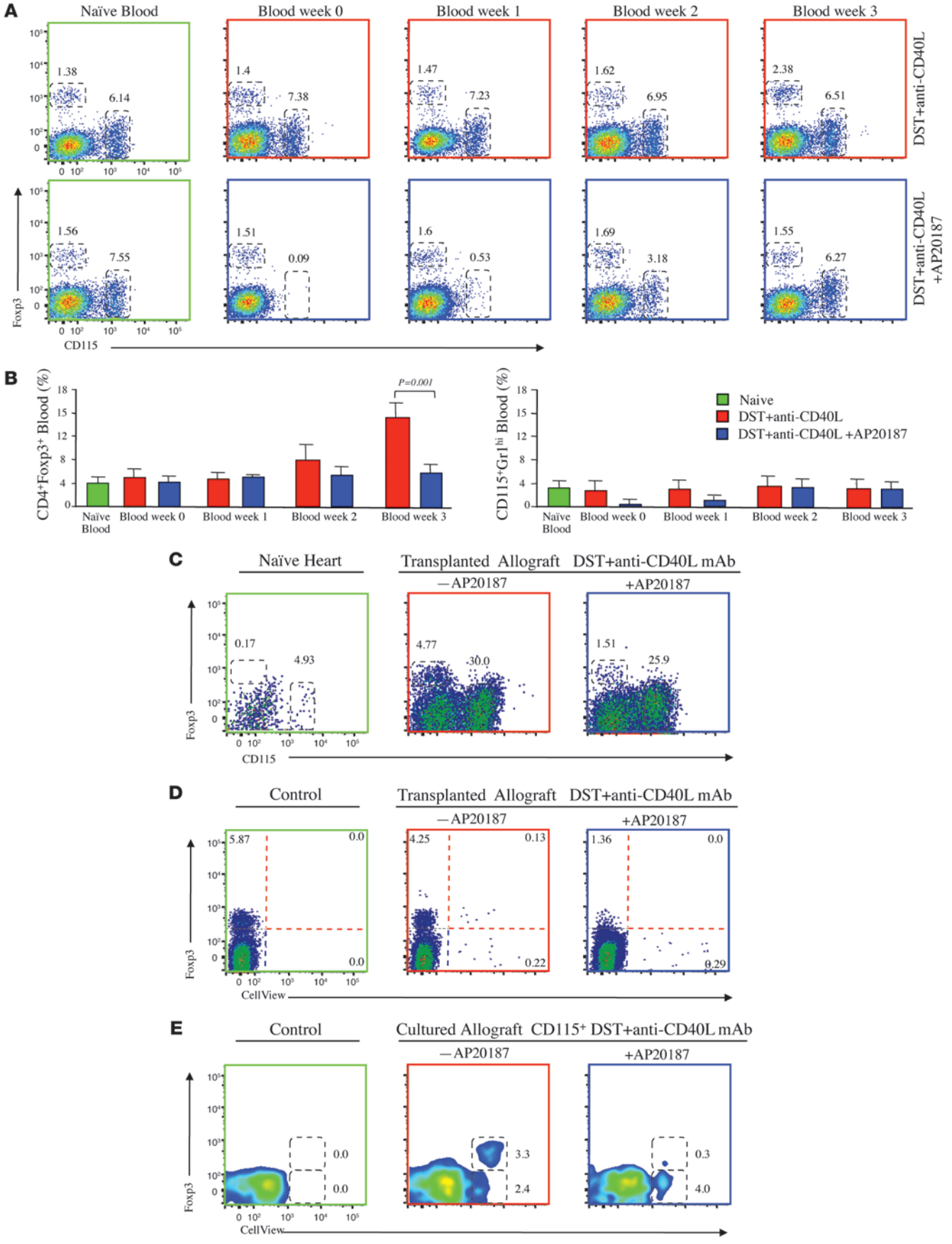
A
Naïve Blood
Blood week 0
Blood week 1
Blood week 2
Blood week 3
DST+anti-CD40L
DST+anti-CD40L +AP20187
1.38
6.14
1.4
7.38
1.47
7.23
1.62
6.95
2.38
6.51
1.56
7.55
1.51
0.09
1.6
0.53
1.69
3.18
1.55
6.27
Foxp3
CD115
B
CD4+Foxp3+ Blood (%)
P=0.001
CD115+Gr1hi Blood (%)
Naive
DST+anti-CD40L
DST+anti-CD40L +AP20187
Naïve Blood
Blood week 0
Blood week 1
Blood week 2
Blood week 3
C
Naïve Heart
Transplanted Allograft DST+anti-CD40L mAb
−AP20187
+AP20187
0.17
4.93
4.77
30.0
1.51
25.9
Foxp3
CD115
D
Control
Transplanted Allograft DST+anti-CD40L mAb
−AP20187
+AP20187
5.87
0.0
0.0
4.25
0.13
0.22
1.36
0.0
0.29
Foxp3
CellView
E
Control
Cultured Allograft CD115+ DST+anti-CD40L mAb
−AP20187
+AP20187
0.0
0.0
3.3
2.4
0.3
4.0
Foxp3
CellView

Figure 4
Tolerogenic monocytes are required for Treg development. (**A**) Dot plots show the percentage of Foxp3$^+$ and CD115$^+$ cells in the blood of tolerogen-treated MaFIA/Foxp3 recipients with and without AP20187 over time (n = 5 mice per group). (**B**) Bar graphs indicate the percentage of CD4$^+$Foxp3$^+$ T cells or CD115$^+$Gr1$^+$ monocytes in tolerogen-treated MaFIA/Foxp3 recipients with and without AP20187 treatment over time. Results represent mean ± SEM. P values were determined by Student's t test. (**C**) Dot plots show the percentage of Foxp3$^+$ and CD115$^+$ cells in the allografts of tolerogen-treated MaFIA/Foxp3 recipients with and without AP20187 treatment (n = 5 mice per group) 3 weeks after transplantation. (**D**) Dot plots show induction of Foxp3 in vivo. CD4$^+$Foxp3$^-$ T cells (1×10^6) were labeled with CellVue and adoptively transferred to tolerogen-treated MaFIA/Foxp3 recipient mice on the day of the transplant. Induction of CD4$^+$Foxp3$^+$ T cells was observed in the allografts of recipient mice 3 weeks after transfer (n = 5 mice per group). (**E**) Dot plots show induction of Foxp3 in vitro. CD115$^+$ cells obtained from allografts of tolerogen-treated MaFIA recipients (2.5×10^4 cells/well) 3 weeks after transplantation were sorted and cultured with CD4$^+$Foxp3$^-$ T cells (5×10^4 cells/well). Induction of CD4$^+$Foxp3$^+$ T cells was observed after 72 hours. Data are representative of 3 independent experiments.

Historically, transplant immunologists have attempted to develop novel tolerogenic protocols by targeting adaptive immune response mechanisms, based on the observation that T cells are both necessary and sufficient to induce allograft rejection. These mechanisms involve deletion of activated T cells (44) and development of Tregs (45). However, tolerance-inducing protocols cannot rely only on lymphoablative therapy (46, 47). Deletion of activated T cells is necessary, but not sufficient, for induction of transplantation tolerance, since T cells from tolerant recipients are able to reject allografts adoptively transferred into new recipients within 3 weeks after transplantation (48). On the other hand, antigen-specific Tregs do not seem to participate during the early phase of tolerance induction (49), as it takes 3 weeks for Tregs to fully develop in the periphery of tolerant recipients (50, 51). These observations suggest that additional mechanisms of early immune regulation must exist that protect the allograft from being acutely rejected by day 10 after transplant. We hypothesize that in addition to previously described adaptive mechanisms necessary for transplantation tolerance, mechanisms that occur during the innate immune response are also required for the induction of indefinite allograft survival; moreover, we propose that in an ischemic/inflamed tissue environment, as a consequence of the surgical procedures intrinsic to transplantation, suppressive monocytes protect the allograft from multiple rejection pathways.

Recent advances in our understanding of how the immune response is influenced by a variety of antigen-nonspecific events have highlighted the participation of the innate immune system in solid organ transplantation and its critical role in shaping the adaptive immune response (52). In this respect, there is increasing interest in investigating the regulation of the immune response by nonlymphoid cells in transplantation (53), and monocytes seem to play an important role in non-self allorecognition during the innate immune response (54). Inflammatory Gr1$^+$ monocytes are excellent candidates to mediate transplantation tolerance induction for several reasons. In contrast to CD11c^{hi} MHC class II$^+$ DCs, which are absent in the blood (55), significant numbers of Gr1$^+$ monocytes constantly circulate in the blood, expressing intermediate levels of CD11c and MHC class II (56), are rapidly recruited to inflamed tissues (57, 58). Once in the inflamed tissue, Gr1$^+$ monocytes become antiinflammatory monocytes in the presence of IFN-γ (59, 60). We observed that CD115$^+$CD11b$^+$Gr1$^+$ monocytes mediated tolerance though IFN-γ–dependent pathways. Although the protective role of IFN-γ in transplantation tolerance remains controversial, accumulating evidence indicates that the IFN-γ signaling pathway is necessary to achieve indefinite allograft survival. Tolerance to vascularized heart allografts is not induced in *Ifng*$^{-/-}$ recipient mice because of exaggerated expansion of alloreactive effector T lymphocytes (61). These tolerogenic effects of IFN-γ occur within the allograft, which may explain the necessity of CD11b$^+$CD115$^+$Gr1$^+$ bone marrow cells in this anatomic compartment. With regard to the protective role of IFN-γ in the allograft, Heslan et al. described that IFN-γ and NO synthase gene expression are upregulated in infiltrating cells of tolerated heart allografts (62), and this in turn is associated with Treg development at the transplanted site (63). Similarly, Wood and colleagues reported that development of alloantigen-reactive Tregs is impaired in the absence of IFN-γ and iNOS within the allograft (64), and, more recently, Bushell and colleagues reported that alloantigen-activated CD4$^+$ T cells cultured in the presence of IFN-γ promotes the generation of Foxp3$^+$ Tregs capable of preventing allograft rejection following adoptive transfer (65, 66). Therefore, expression of IFN-γ and iNOS in the transplanted graft precedes Treg development and the induction of transplantation tolerance. Consistent with this hypothesis, Vanhove and colleagues recently reported that iNOS-expressing MDSCs are necessary for indefinite allograft survival, are required to stimulate IFN-γ secretion of Tregs, and are present tolerant kidney allografts (27). This seminal work from Vanhove and colleagues also suggests that MDSCs are needed during the maintenance phase of transplantation tolerance (27), which highlights the importance of investigating the role of suppressive monocytes over time.

We conclude that bone marrow–derived CD11b$^+$CD115$^+$Gr1$^+$ monocytes are necessary for transplantation tolerance and that manipulation of MDP suggests possible therapeutic approaches using these precursor cells; our results also indicate the need to identify these cells in humans. Manipulation of monocyte development has already demonstrated promising results during sterile inflammation in mice. Geissler and colleagues recently reported that differentiation of monocytes in the presence of IFN-γ results in the development of tolerogenic macrophages in vivo with T cell–suppressive function (67). Similar results have been obtained with human cells in vitro, in which IFN-γ mediated development of tolerogenic DCs from blood monocytes, with the ability to promote Treg development (68). We suggest that there may be at least 2 populations of tolerogenic monocytes that control the immune response: inflammatory Gr1$^+$ monocytes that suppress antigen-nonspecific responses early after transplantation, and antiinflammatory Gr1$^-$ monocytes that promote Treg development though PD-L1 expression. Potential clinical applications of CD11b$^+$CD115$^+$Gr1$^+$ cells in certain pathological conditions, such as cancer, infectious diseases, sepsis, trauma, bone marrow transplantation, and autoimmunity, have previously been suggested (69). Here we extend their use to transplantation to promote induction of indefinite allograft survival, and although their development in other tolerance-inducing protocols needs to be further investigated, evidence suggests that suppressive monocytes are generated with common immunosuppressive therapeutics, such as glucocorticoids (70). In this respect, new strategies are directed to develop suppressive cells in vitro for immunotherapy in transplantation

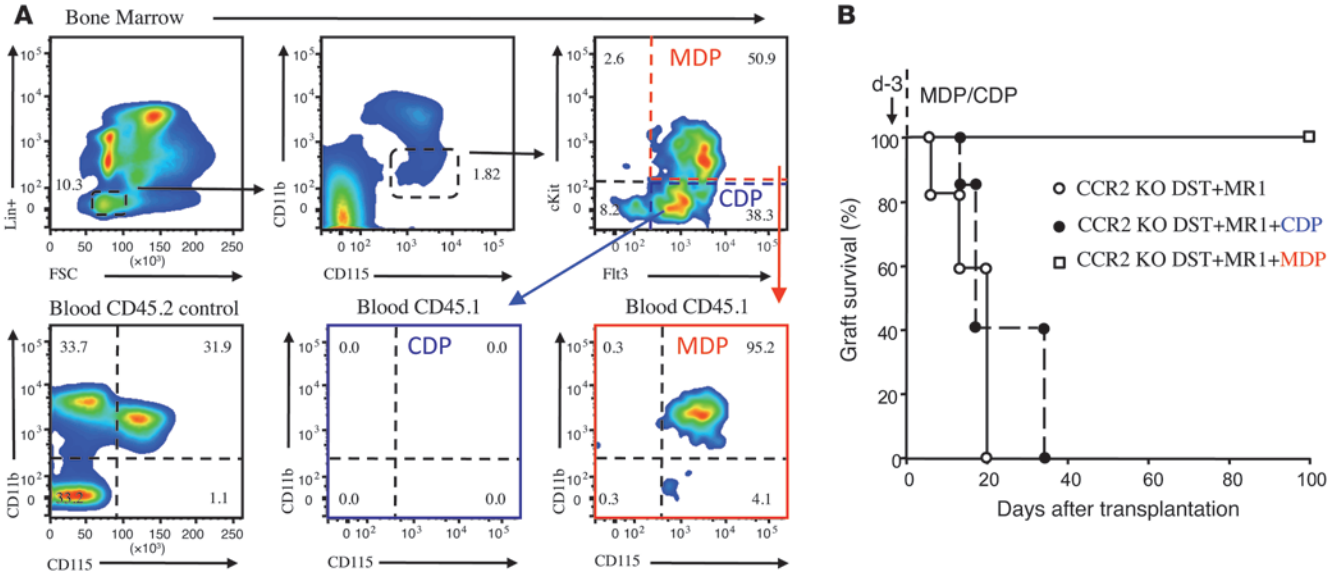

Figure 5

Therapeutic manipulation of tolerogenic monocytes. (**A**) Dot plots show the gating scheme and percentages of MDP and CDP in wild-type bone marrow (top). CD45.1 MDP or CDP was adoptively transferred into CD45.2 mice (bottom). Dot plots indicate that adoptively transferred MDP gave rise to blood circulating CD115$^+$ monocytes, whereas CDP did not. Representative data from 4 independent experiments are shown. Each experiment included at least 3 separately analyzed mice. (**B**) Fully allogeneic vascularized cardiac grafts were rejected by tolerogen-treated *Ccr2*$^{-/-}$ recipient mice receiving 2 × 10^3 bone marrow CDP (n = 5), but accepted by tolerogen-treated *Ccr2*$^{-/-}$ recipient mice receiving 2 × 10^3 bone marrow MDP (n = 5).

(71, 72). Interestingly, Bronte and colleagues have recently identified CCAAT enhancer–binding protein β (C/EBPβ) as a molecular target that can be exploited to generate CD11b$^+$Gr1$^+$ cells with high suppressive function, and have experimental evidence that culturing bone marrow precursors with GM-CSF plus IL-6 generates monocytic CD11b-expressing MDSCs that prolong islet graft survival indefinitely (73).

Methods

Animals. BALB/c and C57BL/6 (*Ccr2*$^{-/-}$, *Ifng*$^{-/-}$, *Ifngr*$^{-/-}$, *Stat1*$^{-/-}$, *Irf1*$^{-/-}$, *Nos2*$^{-/-}$), CD11c-DTR B6.FVB-Tg (Itgax-DTR/GFP), CD11b-DTR B6.FVB-Tg (Itgam-DTR/GFP), and Foxp3-RFP mice 8–12 weeks of age were purchased from The Jackson Laboratory. The C57BL/6-Tg (Csf1r-EGFP-NGFR/FKBP1A/TNFRSF6) 2Bck/J MaFIA mice were from D. Cohen (University of Kentucky, Lexington, Kentucky, USA). *PE*$^{-/-}$ and *FucTIV-VII*$^{-/-}$ mice were a gift from J. Lowe (Case Western University, Cleveland, Ohio, USA). All experiments were performed with age- and sex-matched mice in accordance with Institutional Animal Care and Utilization Committee–approved protocols.

In vivo cell depletion. CD11c-DTR recipients were injected i.p. with 5 ng/g body weight of DT (Sigma-Aldrich) to deplete DCs (12), and CD11b-DTR recipients were treated with 2 doses of DT at 25 ng/g body weight 48 hours apart to deplete monocytes and macrophages (13), 24 hours before transplantation. CD115 MaFIA mice were injected with AP20187, a gift from Ariad Pharmaceuticals. Lyophilized AP20187 was dissolved in 100% ethanol at a concentration of 13.75 mg/ml (1 nM) stock solution and stored at –20°C. MaFIA mice received 0.55 mg/ml of AP20187 containing 4% ethanol, 10% PEG-400, and 1.7% Tween-20 in water. We injected 10 mg/kg AP20187 i.p. on days –5, –4, –3, –2, and –1. For granulocyte depletion, anti-Gr1 mAb clone RB6-8C5 (from R. Coffman, DNAX, Palo Alto, California, USA) was administered (0.5 mg i.v. on days –3, –2, and –1 relative to transplantation; ref. 15). Neutrophil depletion was induced with anti-Gr1 mAb clone 1A8 (BioXcell) at 0.5 mg i.p. on days –3, –2, and –1 relative to transplantation (15). Monocyte/macrophage depletion was performed by i.v. injections of 250 μl liposomes containing clodronate, as previously described (17).

Reagents. anti-CD40L mAb (clone MR1) was from R. Noelle (Dartmouth University, Lebanon, New Hampshire, USA) and purified over protein G or protein A columns (Amersham Pharmacia Biotech). For flow cytometry, anti-CD11b, anti-CD115, anti–Ly-6C/6G (Gr1), anti–C-Kit, anti-FLT3, anti–TER-119, anti–PDCA-1, anti-B220, anti-CD19, anti-CD4, and anti-CD3 were purchased from eBioscience. Clodronate-loaded liposomes were prepared as described previously (13). For bone marrow mobilization, AMD3100 (Sigma-Aldrich) was injected s.c. at 5 mg/kg on day –1 relative to transplantation.

Vascularized heart transplantation. BALB/c hearts were transplanted as fully vascularized heterotopic grafts into C57BL/6 mice as described previously (74). Recipient mice were treated with DST (1 × 10^7 donor splenocytes i.v.) on the day of the transplant together with 500 μg anti-CD40L mAb for tolerance induction (3). DST cells were labeled with ER-Tracker blue-white dye (Invitrogen) to exclude donor monocyte contamination. Graft function was monitored every other day by abdominal palpation. Untreated control mice received hamster IgG in PBS. Rejection was defined as complete cessation of a palpable beat and confirmed by direct visualization at laparotomy.

Cell purification and adoptive transfer. Mice were sacrificed, and the spleen and bone marrow were removed and gently dissociated into single-cell suspensions. Red blood cells were removed by using hypotonic ammonium chloride potassium (ACK) lysis buffer. Cells were placed in complete RPMI medium (RPMI 1640 supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1× nonessential amino acids, and 2 × 10^{-5} M 2-ME). Bone marrow monocytes were stained with 5 μM CFSE (Invitrogen) or CellVue (Sigma-Aldrich) and isolated by sorting on a MoFlo cell sorter (DakoCytomation) to greater than 99% purity, excluding dead cells with DAPI. CD115$^+$CD11b$^+$Gr1^{hi} sorted monocytes (2 × 10^5) were injected i.v. in 300 μl PBS at the indicated times.

Isolation of cardiac allograft leukocytes. Mouse hearts were rinsed in situ with HBSS with 1% heparin. Explanted hearts were cut into small pieces and digested

for 40 minutes at 37°C with 400 U/ml collagenase IV (Sigma-Aldrich), 10 mM HEPES (Cellgro), and 0.01% DNase I (MP Biomedicals) in HBSS (Cellgro). Digested suspensions were passed through a nylon mesh and centrifuged, and the cell pellet was resuspended in 5 ml 45.5% Nycodenz solution (Sigma-Aldrich). Complete DMEM (3 ml) was added to the top of the Nycodenz, and gradient centrifugation was performed (1,700 *g* for 15 minutes at 4°C). The cells at the interface were recovered, washed with complete DMEM, stained, and analyzed by flow cytometry (BD LSR-II; BD Biosciences).

In vitro suppression assay. C57BL/6 mice were sacrificed, spleens were removed and gently dissociated into single-cell suspensions, and red blood cells were removed using hypotonic ACK lysis buffer. Splenocytes were enriched for $CD4^+$ T cells using a $CD4^+$ negative selection kit (R&D Systems). Cells were stained with allophycocyanin-conjugated anti-mouse CD3 and PE-conjugated anti-mouse CD4 mAbs for 30 minutes on ice. $CD3^+CD4^+$ T cells were sorted using FACSVantage DiVa (BD Biosciences) or MoFlo (DakoCytomation). The purity of cells was greater than 99%. Gamma-irradiated (15 Gy) BALB/c ($H\text{-}2^d$) and CBA ($H\text{-}2^k$) splenocytes were depleted of T cells by negative selection with Mouse pan T Dynabeads according to the manufacturer's protocol (Dynal) and used as stimulator cells. Purified $CD11b^+Gr1^+$ allograft cells (2.5×10^4 cells/well) were cultured with BALB/c or CBA T cell–depleted splenocytes (2.5×10^4 cells/well), and CFSE-labeled (5 μM; Invitrogen) C57BL/6 responder $CD3^+CD4^+$ T cells (5×10^4 cells/well) in a final volume of 250 μl complete RPMI medium in U-bottom 96-well plates (Corning). Cells were cultured for 3 days at 37°C in a 5% CO_2 incubator. T cell proliferation was measured by flow cytometric analysis of CFSE dilution.

Quantitative RT-PCR. Total RNA was extracted from purified cells with TRIzol solution (Invitrogen). Reverse transcription was carried out using the Omniscript reverse-transcription system (Qiagen) and random primers. Quantitative PCR was performed with the LightCycler system (Roche) and the SYBR Green PCR kit (Qiagen). All experiments were done at least 3 separate times, and expression of specific genes was normalized and expressed as percentage relative to housekeeping genes (cyclophilin A or GAPDH).

Immunofluorescence microscopy. Transplanted hearts were harvested, subdivided, frozen directly in OCT (Fisher), and stored at –80°C in preparation for immunological studies. Sections of 8 μm were cut using a Leica 1900CM cryomicrotome, fixed, and mounted with Gel/Mount (Biomeda) on polylysine-coated slides. Rabbit anti-mouse iNOS was purchased from Abcam. Phycoerythrin-conjugated rat anti-mouse iNOS was purchased from Jackson Immunoresearch. All slides were mounted with Vectashield (Vector Laboratories) to preserve fluorescence. Images were acquired with a Leica DMRA2 fluorescence microscope (Wetzlar) and a digital Hamamatsu charge-coupled device camera. Separate green, red, and blue images were collected and analyzed with Openlab software (Improvision).

Flow cytometry. Cell washes and Ab dilutions were performed in PBS plus 1% BSA at 4°C. Flow cytometric analysis was performed on LSR II (BD Biosciences) and analyzed with FlowJo (Tree Star). Results are expressed as percentage of cells staining above background, and mAbs were titered at regular intervals during the course of these studies to ensure that saturating concentrations were used.

Statistics. Differences between graft survival rates were assessed by Kaplan-Meier survival analysis with StatView software. Differences between cell numbers and percentages were assessed by 2-tailed Student's *t* test analysis with StatView software. *P* values of 0.05 or less were considered statistically significant.

Acknowledgments

We thank Peter Heeger, Andres Hidalgo (Mount Sinai School of Medicine), and Bernard Vanhove (Université de Nantes) for critical review of the manuscript and Paul Frenette (Mount Sinai School of Medicine) for reagents. We acknowledge the technical contributions of Dan Chen and Jorge Fernández. This work was supported by Programa Ramón y Cajal grant RYC-2006-1588, Ministerio de Educación y Ciencia grant SAF2007-63579, Programa José Castillejo grant JC2008-00065, and Programa de Investigación de Grupos Emergentes del ISCIII (to J.C. Ochando), as well as by NIH R01 grants AI-41428 and AI-72039 and the Emerald Foundation (to J.S. Bromberg).

Received for publication November 2, 2009, and accepted in revised form May 5, 2010.

Address correspondence to: Jordi C. Ochando, Inmunología de Trasplantes, Instituto de Salud Carlos III, 28220 Madrid, Spain. Phone: 34.918223274; Fax: 34.918223269; E-mail: jochando@isciii.es.

1. Lechler RI, Sykes M, Thomson AW, Turka LA. Organ transplantation--how much of the promise has been realized? *Nat Med*. 2005;11(6):605–613.
2. Parker DC, et al. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proc Natl Acad Sci U S A*. 1995;92(21):9560–9564.
3. Hancock WW, Sayegh MH, Zheng XG, Peach R, Linsley PS, Turka LA. Costimulatory function and expression of CD40 ligand, CD80, and CD86 in vascularized murine cardiac allograft rejection. *Proc Natl Acad Sci U S A*. 1996;93(24):13967–13972.
4. Kirk AD, et al. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nat Med*. 1999;5(6):686–693.
5. Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi AB. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat Med*. 2000;6(1):114.
6. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*. 1995;155(3):1151–1164.
7. Haudebourg T, Dugast AS, Coulon F, Usal C, Triebel F, Vanhove B. Depletion of LAG-3 positive cells in cardiac allograft reveals their role in rejection and tolerance. *Transplantation*. 2007;84(11):1500–1506.
8. Niimi M, Hara M, Witzke O, Morris PJ, Wood KJ. Donor resting B cells induce indefinite prolongation of fully allogeneic cardiac grafts when delivered with anti-immunoglobulin-D monoclonal antibody: evidence for tolerogenicity of donor resting B cells in vivo. *Transplantation*. 1998;66(12):1786–1792.
9. Yu G, Xu X, Vu MD, Kilpatrick ED, Li XC. NK cells promote transplant tolerance by killing donor antigen-presenting cells. *J Exp Med*. 2006; 203(8):1851–1858.
10. Seino KI, et al. Requirement for natural killer T (NKT) cells in the induction of allograft tolerance. *Proc Natl Acad Sci U S A*. 2001;98(5):2577–2581.
11. Yamada A, et al. Recipient MHC class II expression is required to achieve long-term survival of murine cardiac allografts after costimulatory blockade. *J Immunol*. 2001;167(10):5522–5526.
12. Duffield JS, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest*. 2005;115(1):56–65.
13. Jung S, et al. In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. *Immunity*. 2002;17(2):211–220.
14. Sunderkotter C, et al. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol*. 2004;172(7):4410–4417.
15. Daley JM, Thomay AA, Connolly MD, Reichner JS, Albina JE. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol*. 2008;83(1):64–70.
16. El-Sawy T, Belperio JA, Strieter RM, Remick DG, Fairchild RL. Inhibition of polymorphonuclear leukocyte-mediated graft damage synergizes with short-term costimulatory blockade to prevent cardiac allograft rejection. *Circulation*. 2005;112(3):320–331.
17. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods*. 1994;174(1–2):83–93.
18. Burnett SH, et al. Conditional macrophage ablation in transgenic mice expressing a Fas-based suicide gene. *J Leukoc Biol*. 2004;75(4):612–623.
19. Murayama T, et al. Intraperitoneal administration of anti-c-fms monoclonal antibody prevents initial events of atherogenesis but does not reduce the size of advanced lesions in apolipoprotein E-deficient mice. *Circulation*. 1999;99(13):1740–1746.
20. Serbina NV, Pamer EG. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol*. 2006;7(3):311–317.
21. Broxmeyer HE, et al. Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J Exp Med*. 2005;201(8):1307–1318.

22. Capoccia BJ, Shepherd RM, Link DC. G-CSF and AMD3100 mobilize monocytes into the blood that stimulate angiogenesis in vivo through a paracrine mechanism. *Blood*. 2006;108(7):2438–2445.
23. Leon B, Ardavin C. Monocyte migration to inflamed skin and lymph nodes is differentially controlled by L-selectin and PSGL-1. *Blood*. 2008;111(6):3126–3130.
24. Maly P, et al. The alpha(1,3)fucosyltransferase Fuc-TVII controls leukocyte trafficking through an essential role in L-, E-, and P-selectin ligand biosynthesis. *Cell*. 1996;86(4):643–653.
25. Zhu B, et al. CD11b+Ly-6C(hi) suppressive monocytes in experimental autoimmune encephalomyelitis. *J Immunol*. 2007;179(8):5228–5237.
26. Mazzoni A, et al. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *J Immunol*. 2002;168(2):689–695.
27. Dugast AS, et al. Myeloid-derived suppressor cells accumulate in kidney allograft tolerance and specifically suppress effector T cell expansion. *J Immunol*. 2008;180(12):7898–7906.
28. Bronte V, et al. IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. *J Immunol*. 2003;170(1):270–278.
29. Gallina G, et al. Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. *J Clin Invest*. 2006;116(10):2777–2790.
30. Dolcetti L, et al. Hierarchy of immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF. *Eur J Immunol*. 2010;40(1):22–35.
31. Youn JI, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J Immunol*. 2008;181(8):5791–5802.
32. Wang L, Han R, Hancock WW. Programmed cell death 1 (PD-1) and its ligand PD-L1 are required for allograft tolerance. *Eur J Immunol*. 2007; 37(10):2983–2990.
33. Sandner SE, et al. Role of the programmed death-1 pathway in regulation of alloimmune responses in vivo. *J Immunol*. 2005;174(6):3408–3415.
34. Wang L, Pino-Lagos K, de Vries VC, Guleria I, Sayegh MH, Noelle RJ. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells. *Proc Natl Acad Sci U S A*. 2008;105(27):9331–9336.
35. Huang B, et al. Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res*. 2006;66(2):1123–1131.
36. Fogg DK, et al. A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science*. 2006;311(5757):83–87.
37. Naik SH, et al. Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. *Nat Immunol*. 2007;8(11):1217–1226.
38. Onai N, Obata-Onai A, Schmid MA, Ohteki T, Jarrossay D, Manz MG. Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. *Nat Immunol*. 2007;8(11):1207–1216.
39. Morelli AE, Thomson AW. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat Rev Immunol*. 2007;7(8):610–621.
40. Steinman RM, Banchereau J. Taking dendritic cells into medicine. *Nature*. 2007;449(7161):419–426.
41. Gabrilovich DI, et al. The terminology issue for myeloid-derived suppressor cells. *Cancer Res*. 2007; 67(1):425.
42. Serafini P, et al. Derangement of immune responses by myeloid suppressor cells. *Cancer Immunol Immunother*. 2004;53(2):64–72.
43. Swirski FK, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science*. 2009;325(5940):612–616.
44. Wells AD, et al. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat Med*. 1999;5(11):1303–1307.
45. Wood KJ, Sakaguchi S. Regulatory T cells in transplantation tolerance. *Nat Rev Immunol*. 2003; 3(3):199–210.
46. Wu Z, et al. Homeostatic proliferation is a barrier to transplantation tolerance. *Nat Med*. 2004; 10(1):87–92.
47. Auchincloss H. No tolerance for depletion. *Nat Med*. 2004;10(1):21–23.
48. Scully R, Qin S, Cobbold S, Waldmann H. Mechanisms in CD4 antibody-mediated transplantation tolerance: kinetics of induction, antigen dependency and role of regulatory T cells. *Eur J Immunol*. 1994;24(10):2383–2392.
49. Kim JI, et al. Regulatory T-cell counter-regulation by innate immunity is a barrier to transplantation tolerance. *Am J Transplant*. 2009;9(12):2736–2744.
50. Saitovitch D, Bushell A, Mabbs DW, Morris PJ, Wood KJ. Kinetics of induction of transplantation tolerance with a nondepleting anti-Cd4 monoclonal antibody and donor-specific transfusion before transplantation. A critical period of time is required for development of immunological unresponsiveness. *Transplantation*. 1996; 61(11):1642–1647.
51. Ochando JC, et al. Lymph node occupancy is required for the peripheral development of alloantigen-specific Foxp3+ regulatory T cells. *J Immunol*. 2005;174(11):6993–7005.
52. LaRosa DF, Rahman AH, Turka LA. The innate immune system in allograft rejection and tolerance. *J Immunol*. 2007;178(12):7503–7509.
53. Dugast AS, Vanhove B. Immune regulation by non-lymphoid cells in transplantation. *Clin Exp Immunol*. 2009;156(1):25–34.
54. Zecher D, van Rooijen N, Rothstein DM, Shlomchik WD, Lakkis FG. An innate response to allogeneic nonself mediated by monocytes. *J Immunol*. 2009;183(12):7810–7816.
55. O'Keeffe M, et al. Dendritic cell precursor populations of mouse blood: identification of the murine homologues of human blood plasmacytoid pre-DC2 and CD11c+ DC1 precursors. *Blood*. 2003;101(4):1453–1459.
56. Naik SH, et al. Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nat Immunol*. 2006;7(6):663–671.
57. Palframan RT, et al. Inflammatory chemokine transport and presentation in HEV: a remote control mechanism for monocyte recruitment to lymph nodes in inflamed tissues. *J Exp Med*. 2001; 194(9):1361–1373.
58. Randolph GJ, Inaba K, Robbiani DF, Steinman RM, Muller WA. Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. *Immunity*. 1999;11(6):753–761.
59. Nahrendorf M, et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J Exp Med*. 2007;204(12):3037–3047.
60. Arnold L, et al. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J Exp Med*. 2007;204(5):1057–1069.
61. Konieczny BT, et al. IFN-gamma is critical for long-term allograft survival induced by blocking the CD28 and CD40 ligand T cell costimulation pathways. *J Immunol*. 1998;160(5):2059–2064.
62. Heslan JM, Renaudin K, Thebault P, Josien R, Cuturi MC, Chiffoleau E. New evidence for a role of allograft accommodation in long-term tolerance. *Transplantation*. 2006;82(9):1185–1193.
63. Heslan JM, et al. Accumulation of T cells with potent regulatory properties and restricted Vbeta7-TCR rearrangements in tolerated allografts. *Transplantation*. 2005;80(10):1476–1484.
64. Sawitzki B, Kingsley CI, Oliveira V, Karim M, Herber M, Wood KJ. IFN-gamma production by alloantigen-reactive regulatory T cells is important for their regulatory function in vivo. *J Exp Med*. 2005;201(12):1925–1935.
65. Feng G, et al. Exogenous IFN-gamma ex vivo shapes the alloreactive T-cell repertoire by inhibition of Th17 responses and generation of functional Foxp3+ regulatory T cells. *Eur J Immunol*. 2008;38(9):2512–2527.
66. Feng G, Wood KJ, Bushell A. Interferon-gamma conditioning ex vivo generates CD25+CD62L+Foxp3+ regulatory T cells that prevent allograft rejection: potential avenues for cellular therapy. *Transplantation*. 2008;86(4):578–589.
67. Brem-Exner BG, et al. Macrophages driven to a novel state of activation have anti-inflammatory properties in mice. *J Immunol*. 2008;180(1):335–349.
68. Eljaafari A, Li YP, Miossec P. IFN-gamma, as secreted during an alloresponse, induces differentiation of monocytes into tolerogenic dendritic cells, resulting in FoxP3+ regulatory T cell promotion. *J Immunol*. 2009;183(5):2932–2945.
69. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. 2009;9(3):162–174.
70. Varga G, et al. Glucocorticoids induce an activated, anti-inflammatory monocyte subset in mice that resembles myeloid-derived suppressor cells. *J Leukoc Biol*. 2008;84(3):644–650.
71. De Wilde V, et al. Endotoxin-induced myeloid-derived suppressor cells inhibit alloimmune responses via heme oxygenase-1. *Am J Transplant*. 2009;9(9):2034–2047.
72. Zhou Z, et al. Development and function of myeloid-derived suppressor cells generated from mouse embryonic and hematopoietic stem cells. *Stem Cells*. 2010;28(3):620–632.
73. Marigo I, et al. Tumor-induced tolerance and immune suppression depend on the C/EBPbeta transcription factor [published online ahead of print June 3, 2010]. *Immunity*. doi:10.1016/j.immuni.2010.05.010.
74. Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation*. 1973;16(4):343–350.