



# Monocytic suppressive cells mediate cardiovascular transplantation tolerance in mice

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**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<sup>+</sup>, CD11b<sup>+</sup>, or CD115<sup>+</sup> cells, we identified a tolerogenic role for CD11b<sup>+</sup>CD115<sup>+</sup>Gr1<sup>+</sup> monocytes during the induction of tolerance by costimulatory blockade with CD40L-specific mAb. Early after transplantation, Gr1<sup>+</sup> 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<sup>+</sup>CD115<sup>+</sup>Gr1<sup>+</sup> 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

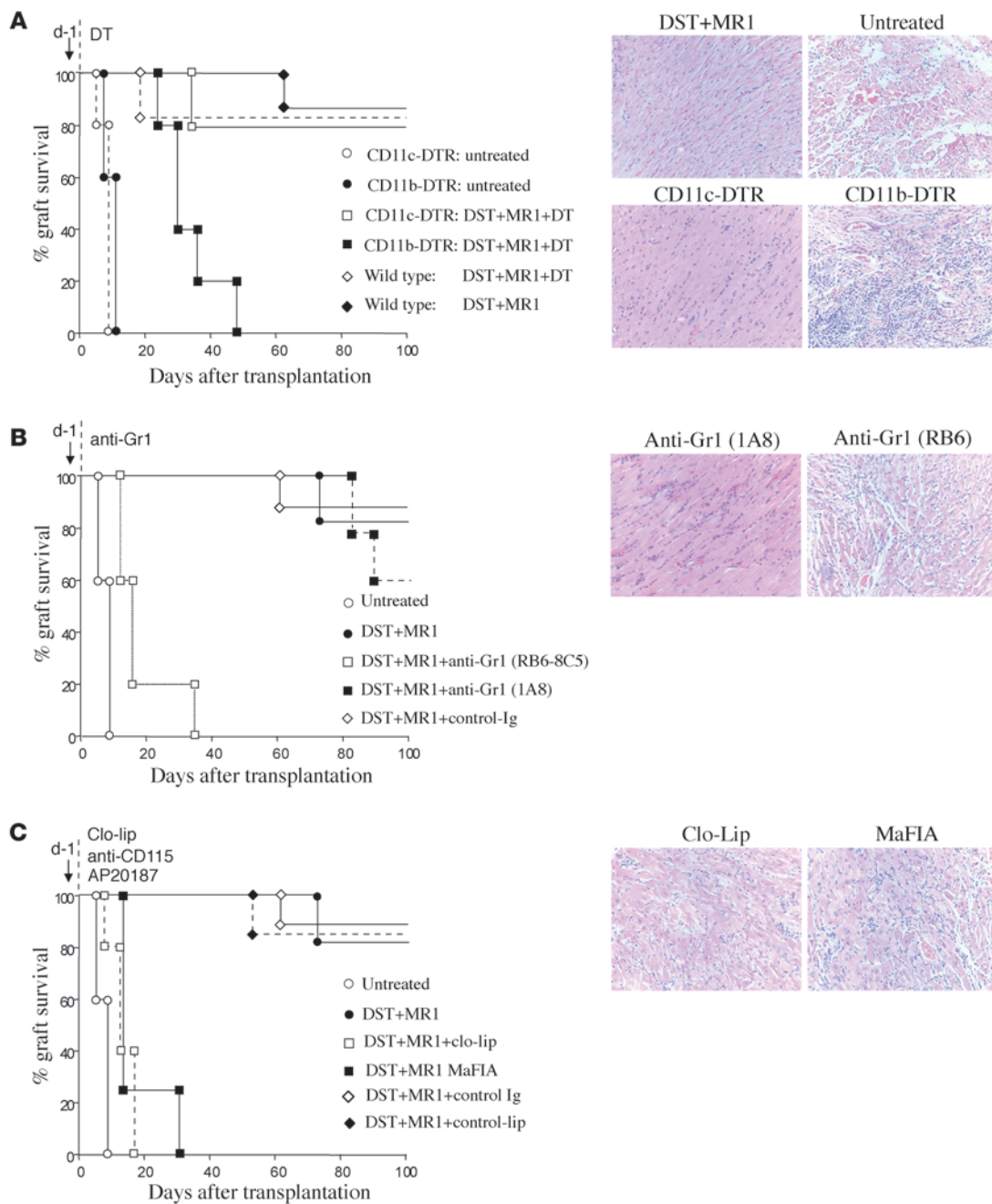
deplete cells *in vivo* and monitor the outcome of the immune response in the absence of the targeted cells. In experimental transplantation, the use of depletional mAbs and knockout or transgenic mouse strains has defined tolerogenic roles for Tregs (6), T cells (7), B cells (8), NK cells (9), and NKT 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<sup>+</sup> 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<sup>+</sup>CD115<sup>+</sup>Gr1<sup>+</sup> monocytes as suppressive cells that inhibit the immune response early after transplantation. Using adoptive

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**Figure 1**

CD11b<sup>+</sup>CD115<sup>+</sup>Gr1<sup>+</sup> 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,  $\times 40$ .

transfer studies in recipients with reduced numbers of circulating CD11b<sup>+</sup>CD115<sup>+</sup>Gr1<sup>+</sup> monocytes, we further identified the anatomic locations and mechanisms of action by which these cells exert their immune regulatory function, which include antigen-non-specific 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<sup>+</sup>CD115<sup>+</sup>Gr1<sup>+</sup> 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<sup>+</sup> DCs and CD11b<sup>+</sup> monocytes, macrophages, and neutrophils, respectively (12, 13). Vascularized BALB/c (H-2<sup>d</sup>) hearts were transplanted into fully allogeneic C57BL/6 (H-2<sup>b</sup>) CD11c-DTR or CD11b-DTR mice, and recipients were either left untreated for acute rejection or administered DST ( $1 \times 10^7$  cells i.v.) together with 500  $\mu$ g 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.

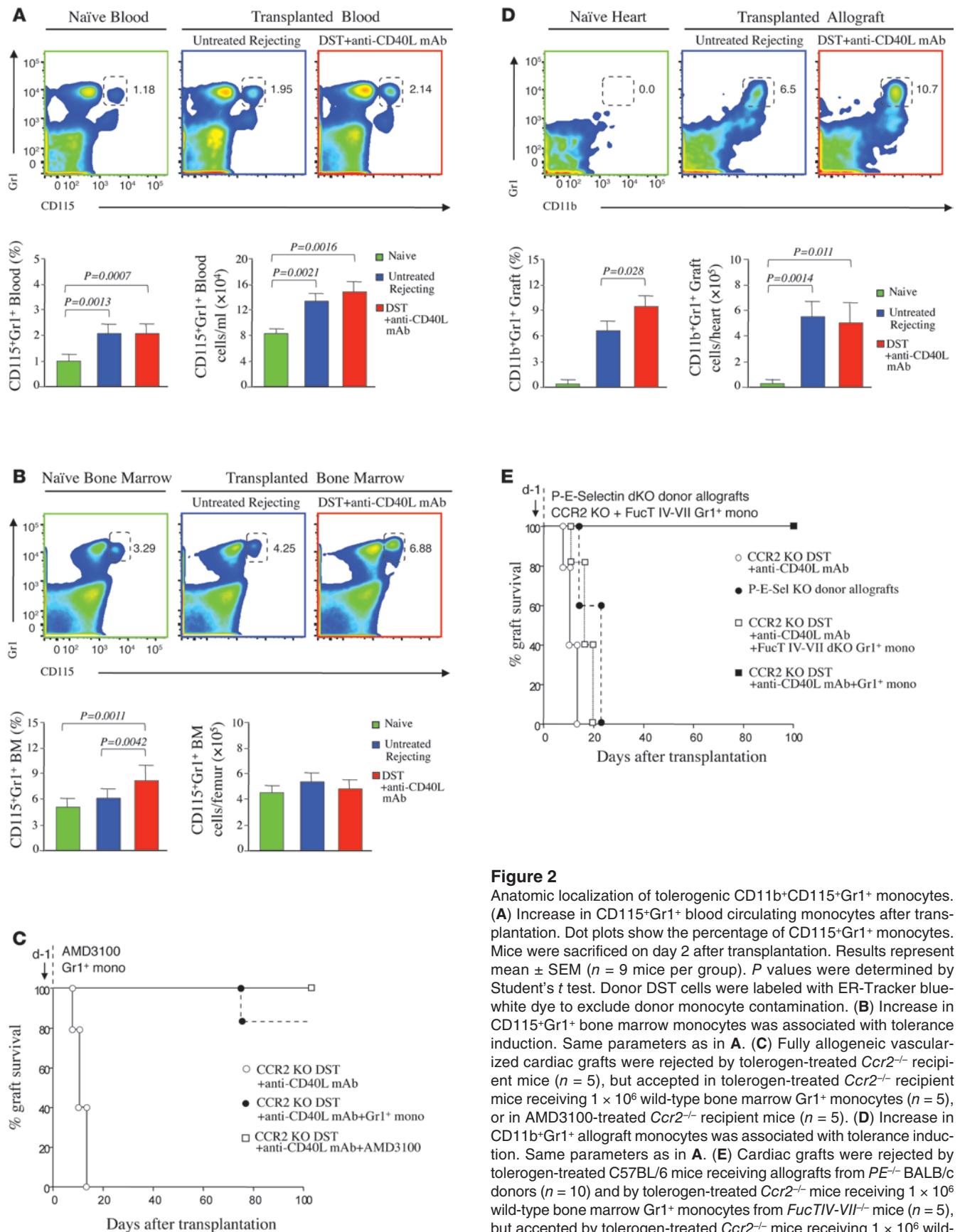
There are 2 main subsets of circulating monocytes that are characterized by their expression of CD11c and Gr1. CD11c<sup>+</sup> monocytes are Gr1<sup>-</sup>, and CD11c<sup>-</sup> monocytes are Gr1<sup>+</sup> (14). Depletion of CD11c did not prevent tolerance induction (Figure 1A), which suggested that CD11b<sup>+</sup>Gr1<sup>+</sup> monocytes might mediate tolerance induction. To investigate the tolerogenic role of Gr1<sup>+</sup> monocytes during tolerance induction, depletion doses of the anti-Gr1 mAb clone RB6-8C5 were administered to tolerogen-treated wild-type recipients. The results indicated that depletion of Gr1<sup>+</sup> cells prevented tolerance (Figure 1B and Supplemental Figure 1). Since anti-Gr1 mAb clone RB6-8C5 depletes both Gr1<sup>+</sup> monocytes and neutrophils under steady-state conditions (15), the tolerogenic role of Gr1<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup>CD115<sup>+</sup>Gr1<sup>+</sup> monocytes.* The anatomic location of CD115<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup> monocytes and their trafficking requirements were next investigated. Monocytes are blood-circulating cells, and the results showed an increase of

CD115<sup>+</sup>Gr1<sup>+</sup> monocytes in the blood of transplanted recipients (Figure 2A). This suggested that CD115<sup>+</sup>Gr1<sup>+</sup> monocytes were being generated in the bone marrow, which was confirmed by the observed increase of CD115<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup> monocytes in the bone marrow of tolerized recipients (Figure 2B). To investigate the necessity of Gr1<sup>+</sup> monocyte mobilization from the bone marrow during tolerance induction, we used CCR2-deficient (*Ccr2*<sup>-/-</sup>) mice, which have a 70% reduction in blood circulating Gr1<sup>+</sup> 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<sup>+</sup>Gr1<sup>+</sup> monocytes in tolerogen-treated *Ccr2*<sup>-/-</sup> 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<sup>+</sup> monocytes was necessary for tolerance induction. To investigate this hypothesis, Gr1<sup>+</sup> monocytes were mobilized in tolerogen-treated *Ccr2*<sup>-/-</sup> 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<sup>+</sup> monocytes restored indefinite allograft survival in tolerogen-treated *Ccr2*<sup>-/-</sup> recipients. Because treatment with AMD3100 mobilizes granulocytes in addition to Gr1<sup>+</sup> monocytes from the bone marrow, wild-type bone marrow CD115<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup> monocytes (Supplemental Figure 2B) were adoptively transferred to *Ccr2*<sup>-/-</sup> recipients, and graft survival was monitored. Figure 2C indicates that transfer of wild-type bone marrow CD115<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup> monocytes also induced tolerance in tolerogen-treated *Ccr2*<sup>-/-</sup> recipients. Together, these results showed that mobilization of CD115<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup> from bone marrow to blood was necessary for tolerance induction.

Further analysis demonstrated an increase in CD11b<sup>+</sup>Gr1<sup>+</sup> monocytes in the allografts of tolerogen-treated recipients (Figure 2D), suggestive of Gr1<sup>+</sup> monocyte trafficking from the blood to the transplanted organ during tolerance induction. To investigate the necessity of CD115<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup> 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*<sup>-/-</sup>) donors. The results indicated that *PE*<sup>-/-</sup> allografts were rapidly rejected, accompanied by a significant decrease in Gr1<sup>+</sup> 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<sup>+</sup>Gr1<sup>+</sup> 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*<sup>-/-</sup> recipients were adoptively transferred with CD115<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup> monocytes from fucosyltransferase IV-VII double-deficient (*FucTIV-VII*<sup>-/-</sup>) 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-VII*<sup>-/-</sup> 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<sup>+</sup>Gr1<sup>+</sup> monocytes, *FucTIV-VII*<sup>-/-</sup> Gr1<sup>+</sup> 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<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup> monocyte trafficking into the grafted heart was necessary for tolerance induction.



**Figure 2**

Anatomic localization of tolerogenic CD11b<sup>+</sup>CD115<sup>+</sup>Gr1<sup>+</sup> monocytes. **(A)** Increase in CD115<sup>+</sup>Gr1<sup>+</sup> blood circulating monocytes after transplantation. Dot plots show the percentage of CD115<sup>+</sup>Gr1<sup>+</sup> 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<sup>+</sup>Gr1<sup>+</sup> 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*<sup>-/-</sup> recipient mice (*n* = 5), but accepted in tolerogen-treated *Ccr2*<sup>-/-</sup> recipient mice receiving 1 × 10<sup>6</sup> wild-type bone marrow Gr1<sup>+</sup> monocytes (*n* = 5), or in AMD3100-treated *Ccr2*<sup>-/-</sup> recipient mice (*n* = 5). **(D)** Increase in CD11b<sup>+</sup>Gr1<sup>+</sup> 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*<sup>-/-</sup> BALB/c donors (*n* = 10) and by tolerogen-treated *Ccr2*<sup>-/-</sup> mice receiving 1 × 10<sup>6</sup> wild-type bone marrow Gr1<sup>+</sup> monocytes from *FucTIV-VII*<sup>+/-</sup> mice (*n* = 5), but accepted by tolerogen-treated *Ccr2*<sup>-/-</sup> mice receiving 1 × 10<sup>6</sup> wild-type bone marrow Gr1<sup>+</sup> monocytes (*n* = 5).



*Mechanisms of action of tolerogenic CD11b<sup>+</sup>CD115<sup>+</sup>Gr1<sup>+</sup> monocytes.* Suppressive CD11b<sup>+</sup>Gr1<sup>+</sup> monocytes have been shown in other model systems to mediate their inhibitory function through IFN- $\gamma$ -dependent pathways (25, 26). To investigate the mechanisms by which CD11b<sup>+</sup>Gr1<sup>+</sup> monocytes exert their tolerogenic function in transplantation, CD115<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup> bone marrow monocytes from IFN- $\gamma$ -deficient (*Ifng*<sup>-/-</sup>) and IFN- $\gamma$  receptor-deficient (*Ifngr*<sup>-/-</sup>) mice were adoptively transferred into tolerogen-treated *Ccr2*<sup>-/-</sup> mice, and allograft survival was monitored. Tolerance was induced by adoptively transferred bone marrow CD11b<sup>+</sup>Gr1<sup>+</sup> monocytes from *Ifng*<sup>-/-</sup> mice. In contrast, tolerance was not induced with adoptively transferred CD11b<sup>+</sup>Gr1<sup>+</sup> bone marrow monocytes from *Ifngr*<sup>-/-</sup> mice (Figure 3A). These results suggested that CD11b<sup>+</sup>Gr1<sup>+</sup> monocytes mediated their suppressive effect through IFN- $\gamma$ R signaling. NO, one of the downstream pathways for IFN- $\gamma$ R, is expressed in CD11b<sup>+</sup>Gr1<sup>+</sup>-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<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup> monocytes during transplantation tolerance and found that tolerance was not induced in tolerogen-treated *Ccr2*<sup>-/-</sup> recipients that were transferred with iNOS-deficient (*Nos2*<sup>-/-</sup>) bone marrow monocytes (Figure 3A). Moreover, monocytes deficient in molecules that participate in the signaling pathway between IFN- $\gamma$ R and iNOS (STAT-1 and IRF-1) were also unable to restore tolerance in tolerogen-treated *Ccr2*<sup>-/-</sup> 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<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup> 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<sup>+</sup>Gr1<sup>+</sup> 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<sup>d</sup>) or third-party CBA (H-2<sup>k</sup>) as stimulators and syngeneic C57BL/6 T cells as responders, sorted CD11b<sup>+</sup>Gr1<sup>+</sup> monocytes from the allograft of tolerogen-treated recipients suppressed T cell proliferation in an antigen-nonspecific manner, whereas CD11b<sup>+</sup>Gr1<sup>+</sup> 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<sup>+</sup>Gr1<sup>+</sup> monocytes in tolerized allografts did not express PD-L1. Interestingly, the CD11b<sup>+</sup>Gr1<sup>-</sup> 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).

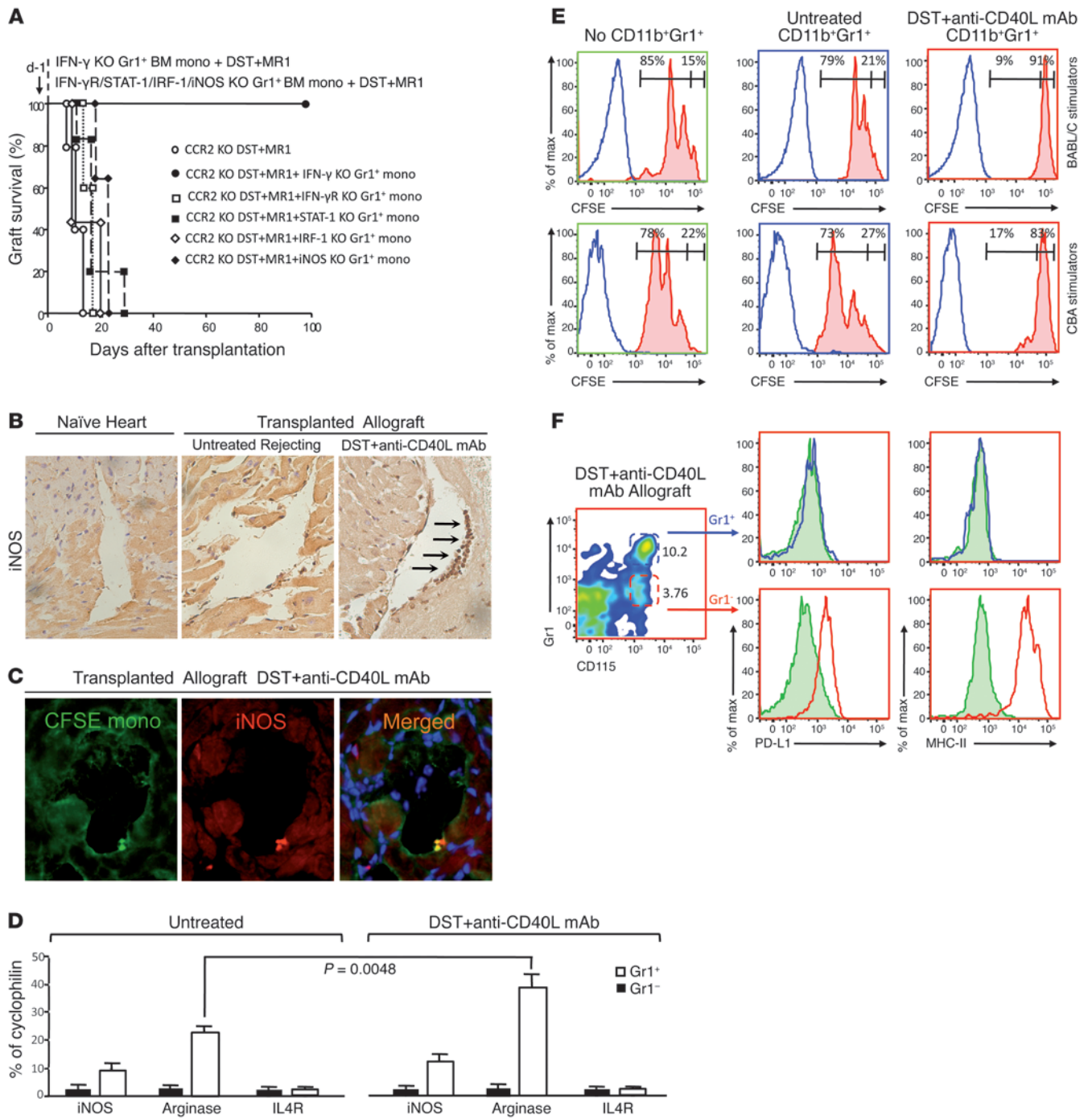
*Tolerogenic monocytes are required for Treg development.* Monocytic suppressor cells expressing CD115 have been recently shown to mediate Treg development in vivo (35). To monitor Treg development by monocytic suppressor cells in vivo, we crossed CD115-GFP MaFIA mice (H-2<sup>b</sup>) with FoxP3-RFP mice (H-2<sup>b</sup>) to obtain mice in which Tregs could be monitored following monocyte depletion. BALB/c (H-2<sup>d</sup>) hearts were transplanted into fully allogeneic tolerogen-treated MaFIA/Foxp3 (H-2<sup>b</sup>) 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,  $1 \times 10^6$  CD4<sup>+</sup>Foxp3<sup>-</sup> 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<sup>+</sup>Foxp3<sup>+</sup> 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<sup>+</sup>Foxp3<sup>-</sup> T cells to become Foxp3<sup>+</sup>. 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<sup>+</sup>Foxp3<sup>-</sup> 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*<sup>-/-</sup> 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*<sup>-/-</sup> recipients that received CDP (Figure 5B). In contrast, tolerance was induced in *Ccr2*<sup>-/-</sup> 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.

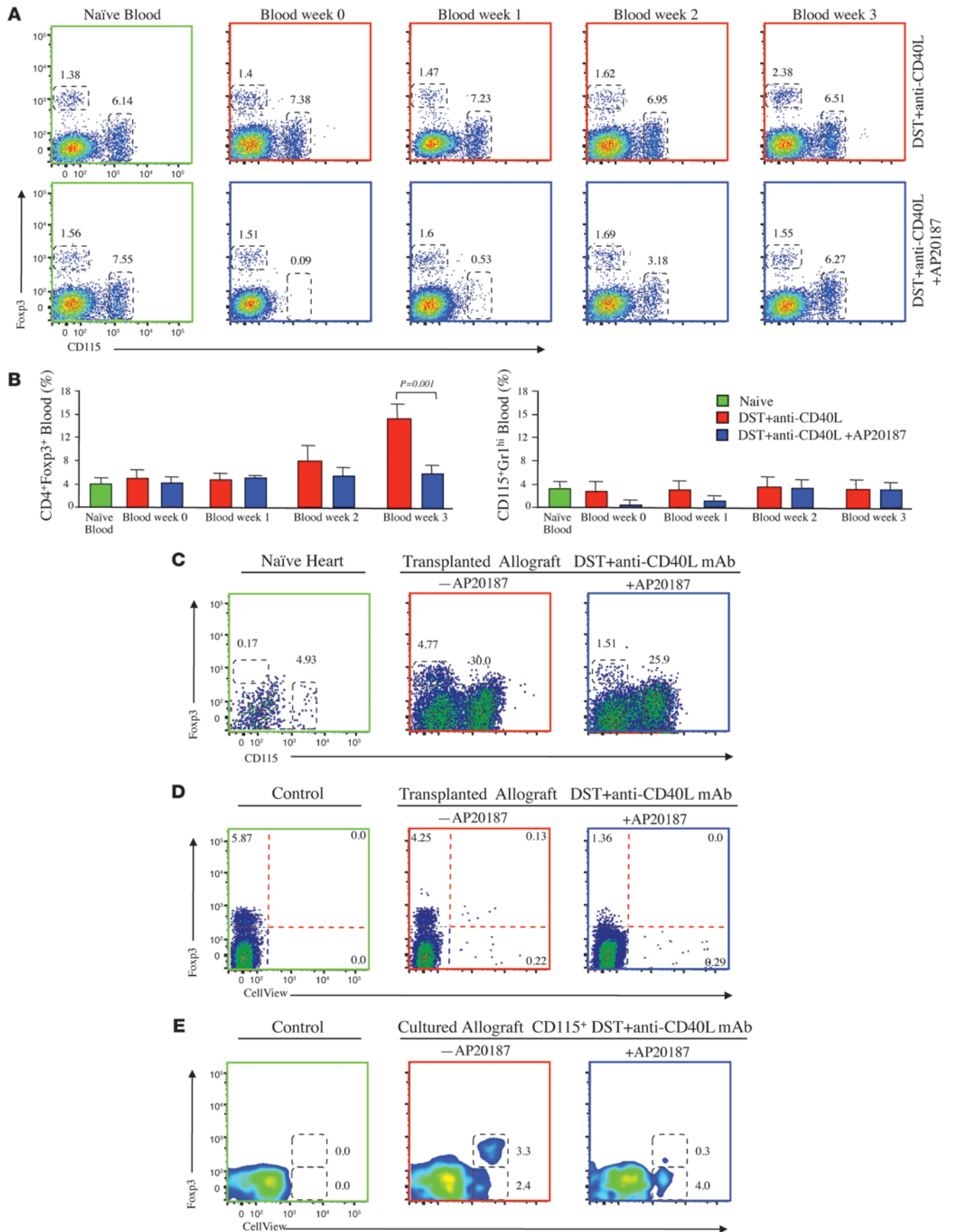
## Discussion

We identified CD11b<sup>+</sup>CD115<sup>+</sup>Gr1<sup>+</sup> 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<sup>+</sup>CD115<sup>+</sup>Gr1<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup>CD115<sup>+</sup>Gr1<sup>+</sup> 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<sup>+</sup>CD115<sup>+</sup>Gr1<sup>+</sup> monocytes. **(A)** Cardiac allografts were accepted by tolerogen-treated *Ccr2*<sup>-/-</sup> mice receiving  $1 \times 10^6$  bone marrow Gr1<sup>+</sup> monocytes from *Irfng*<sup>-/-</sup> mice, but rejected by mice receiving  $1 \times 10^6$  bone marrow Gr1<sup>+</sup> monocytes from *Irfng*<sup>-/-</sup>, *Stat1*<sup>-/-</sup>, *Irf1*<sup>-/-</sup>, or *Nos2*<sup>-/-</sup> mice ( $n = 5$  per group). **(B)** Representative images of immunohistochemical analysis of iNOS<sup>+</sup> cells around graft endothelial cells 2 days after transplantation ( $n = 5$  mice per group). Original magnification,  $\times 40$ . **(C)** Fluorescent immunohistochemistry of CFSE-labeled adoptively transferred monocytes stained with iNOS in the allograft ( $n = 2 \pm 0.3$  cells/vessel; 20 vessels/heart;  $n = 3$  mice). Original magnification,  $\times 40$ . **(D)** Real-time RT-PCR of *iNOS*, *arginase*, and *IL-4R* expression in CD11b<sup>+</sup>Gr1<sup>+</sup> 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<sup>+</sup>CD4<sup>+</sup> T cells from C57BL/6 mice were sorted and labeled with CFSE, and  $5 \times 10^4$  cells/well were cultured with CD11b<sup>+</sup>Gr1<sup>+</sup> allograft monocytes ( $2.5 \times 10^4$  cells/well) in the presence of BALB/c or CBA APC ( $2.5 \times 10^4$  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<sup>+</sup>Gr1<sup>+</sup> and CD115<sup>+</sup>Gr1<sup>-</sup> monocytes in the allografts of tolerant mice. Mice were sacrificed on day 2 after transplantation. Results are mean  $\pm$  SEM ( $n = 5$  mice). Donor DST cells were labeled with ER-Tracker blue-white dye to exclude donor monocyte contamination.





#### Figure 4

Tolerogenic monocytes are required for Treg development. (A) Dot plots show the percentage of Foxp3<sup>+</sup> and CD115<sup>+</sup> 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<sup>+</sup>Foxp3<sup>+</sup> T cells or CD115<sup>+</sup>Gr1<sup>+</sup> monocytes in tolerogen-treated MaFIA/Foxp3 recipients with and without AP20187 treatment over time. Results represent mean  $\pm$  SEM.  $P$  values were determined by Student's  $t$  test. (C) Dot plots show the percentage of Foxp3<sup>+</sup> and CD115<sup>+</sup> 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<sup>+</sup>Foxp3<sup>-</sup> T cells ( $1 \times 10^6$ ) were labeled with CellVue and adoptively transferred to tolerogen-treated MaFIA/Foxp3 recipient mice on the day of the transplant. Induction of CD4<sup>+</sup>Foxp3<sup>+</sup> 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<sup>+</sup> cells obtained from allografts of tolerogen-treated MaFIA recipients ( $2.5 \times 10^4$  cells/well) 3 weeks after transplantation were sorted and cultured with CD4<sup>+</sup>Foxp3<sup>-</sup> T cells ( $5 \times 10^4$  cells/well). Induction of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells was observed after 72 hours. Data are representative of 3 independent experiments.

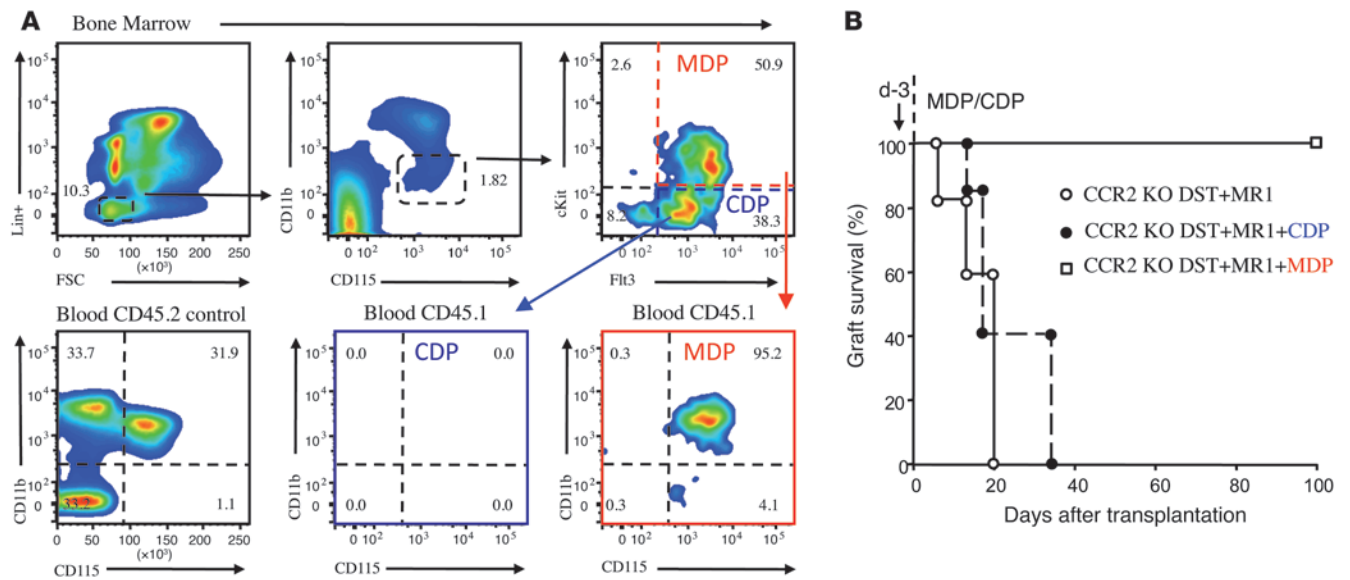
Historically, transplant immunologists have attempted to develop novel tolerogenic protocols by targeting adaptive immune response mechanisms, based on the observation that T cells are both necessary and sufficient to induce allograft rejection. These mechanisms involve deletion of activated T cells (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<sup>+</sup> monocytes are excellent candidates to mediate transplantation tolerance induction for several reasons. In contrast to CD11c<sup>hi</sup> MHC class II<sup>+</sup> DCs, which are absent in the blood (55), significant numbers of Gr1<sup>+</sup> 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 tis-

sue, Gr1<sup>+</sup> monocytes become antiinflammatory monocytes in the presence of IFN- $\gamma$  (59, 60). We observed that CD115<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup> monocytes mediated tolerance through IFN- $\gamma$ -dependent pathways. Although the protective role of IFN- $\gamma$  in transplantation tolerance remains controversial, accumulating evidence indicates that the IFN- $\gamma$  signaling pathway is necessary to achieve indefinite allograft survival. Tolerance to vascularized heart allografts is not induced in *Ifng*<sup>-/-</sup> recipient mice because of exaggerated expansion of alloreactive effector T lymphocytes (61). These tolerogenic effects of IFN- $\gamma$  occur within the allograft, which may explain the necessity of CD11b<sup>+</sup>CD115<sup>+</sup>Gr1<sup>+</sup> bone marrow cells in this anatomic compartment. With regard to the protective role of IFN- $\gamma$  in the allograft, Heslan et al. described that IFN- $\gamma$  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- $\gamma$  and iNOS within the allograft (64), and, more recently, Bushell and colleagues reported that alloantigen-activated CD4<sup>+</sup> T cells cultured in the presence of IFN- $\gamma$  promotes the generation of Foxp3<sup>+</sup> Tregs capable of preventing allograft rejection following adoptive transfer (65, 66). Therefore, expression of IFN- $\gamma$  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- $\gamma$  secretion of Tregs, and are present in 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<sup>+</sup>CD115<sup>+</sup>Gr1<sup>+</sup> 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- $\gamma$  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- $\gamma$  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<sup>+</sup> monocytes that suppress antigen-nonspecific responses early after transplantation, and antiinflammatory Gr1<sup>-</sup> monocytes that promote Treg development through PD-L1 expression. Potential clinical applications of CD11b<sup>+</sup>CD115<sup>+</sup>Gr1<sup>+</sup> 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<sup>+</sup> 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*<sup>-/-</sup> recipient mice receiving  $2 \times 10^3$  bone marrow CDP ( $n = 5$ ), but accepted by tolerogen-treated *Ccr2*<sup>-/-</sup> recipient mice receiving  $2 \times 10^3$  bone marrow MDP ( $n = 5$ ).

(71, 72). Interestingly, Bronte and colleagues have recently identified CCAAT enhancer-binding protein  $\beta$  (C/EBP $\beta$ ) as a molecular target that can be exploited to generate CD11b<sup>+</sup>Gr1<sup>+</sup> 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*<sup>-/-</sup>, *Ifng*<sup>-/-</sup>, *Ifngr*<sup>-/-</sup>, *Stat1*<sup>-/-</sup>, *Irf1*<sup>-/-</sup>, *Nos2*<sup>-/-</sup>), CD11c-DTR B6.FVB-Tg (*Itgax*-DTR/GFP), CD11b-DTR B6.FVB-Tg (*Itgax*-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*<sup>-/-</sup> and *FucTIV-VII*<sup>-/-</sup> 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  $\mu$ l 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 ( $1 \times 10^7$  donor splenocytes i.v.) on the day of the transplant together with 500  $\mu$ g 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  $\mu$ g/ml streptomycin, 1x nonessential amino acids, and  $2 \times 10^{-5}$  M 2-ME). Bone marrow monocytes were stained with 5  $\mu$ M 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<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>hi</sup> sorted monocytes ( $2 \times 10^5$ ) were injected i.v. in 300  $\mu$ l 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<sup>+</sup> T cells using a CD4<sup>+</sup> 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<sup>+</sup>CD4<sup>+</sup> T cells were sorted using FACS Vantage DiVa (BD Biosciences) or MoFlo (DakoCytomation). The purity of cells was greater than 99%. Gamma-irradiated (15 Gy) BALB/c (H-2<sup>d</sup>) and CBA (H-2<sup>k</sup>) splenocytes were depleted of T cells by negative selection with Mouse pan T Dynabeads according to the manufacturer's protocol (Dyna) and used as stimulator cells. Purified CD11b<sup>+</sup>Gr1<sup>+</sup> allograft cells (2.5 × 10<sup>4</sup> cells/well) were cultured with BALB/c or CBA T cell-depleted splenocytes (2.5 × 10<sup>4</sup> cells/well), and CFSE-labeled (5 μM; Invitrogen) C57BL/6 responder CD3<sup>+</sup>CD4<sup>+</sup> T cells (5 × 10<sup>4</sup> 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<sub>2</sub> 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 (Biomed) 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 titrated 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.

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