Recruitment of Foxp3^{+} T regulatory cells mediating allograft tolerance depends on the CCR4 chemokine receptor

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Although certain chemokines and their receptors guide homeostatic recirculation of T cells and others promote recruitment of activated T cells to inflammatory sites, little is known of the mechanisms underlying a third function, migration of Foxp3^{+} regulatory T (T reg) cells to sites where they maintain unresponsiveness. We studied how T reg cells are recruited to cardiac allografts in recipients tolerized with CD154 monoclonal antibody (mAb) plus donor-specific transfusion (DST). Real-time polymerase chain reaction showed that intragraft Foxp3 levels in tolerized recipients were ~100-fold higher than rejecting allografts or allografts associated with other therapies inducing prolonged survival but not tolerance. Foxp3^{+} cells were essential for tolerance because pretransplant thymectomy or peritransplant depletion of CD25^{+} cells prevented long-term survival, as did CD25 mAb therapy in well-functioning allografts after CD154/DST therapy. Analysis of multiple chemokine pathways showed that tolerance was accompanied by intragraft up-regulation of CCR4 and one of its ligands, macrophage-derived chemokine (CCL22), and that tolerance induction could not be achieved in CCR4^{−/−} recipients. We conclude that Foxp3 expression is specifically up-regulated within allografts of mice displaying donor-specific tolerance, that recruitment of Foxp3-expressing T reg cells to an allograft tissue is dependent on the chemokine receptor, CCR4, and that, in the absence of such recruitment, tolerizing strategies such as CD154 mAb therapy are ineffectual.

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Previous studies have demonstrated a role for regulatory T (T reg) cells in promotion of allograft tolerance (1–6). However, little is known concerning the relative importance of naturally occurring T reg cells versus T reg cells induced posttransplantation (post-Tx), the signals responsible for T reg cell generation and function in vivo, and how T reg cells home to key sites. Study of T reg cells in vivo is complicated by a paucity of phenotypic markers to distinguish activated effector T cells from T reg cells post-Tx. CTL antigen-4, glucocorticoid-induced TNF receptor (GITR), and the cytokines TGF-β and IL-10 have each been linked with, but not proven entirely specific for, T reg cells (1, 7–9). In contrast, Foxp3, a gene that encodes a forkhead/winged helix transcription factor Scurfin, is required for T reg cell development and studies support its utility as a marker of T reg cells (10, 11).

Of the multiple forms of costimulation blockade that can promote long-term engraftment in rodent models, only targeting of CD40–CD154 interactions, typically in conjunction with donor-specific transfusion (DST), has proven widely successful in promoting donor-specific tolerance (12–14). Indeed, in the absence of knowledge of the signals required for induction of T reg cells, it is conceivable that some forms of costimulatory blockade may promote long-term allograft survival but prove detrimental to formal tolerance induction. In the case of CD154 mAb-linked tolerance, well-functioning allografts consistently show host mononuclear cell infiltration but an absence of target organ injury, suggesting a potentially active process of regulation within the graft (15). The presence of Foxp3^{+} cells in tolerized grafts after CD4 mAb blockade has been demonstrated in skin allograft

studies (4, 5). However, despite in vitro studies indicating that various chemokine receptors are expressed by human and murine T reg cells, nothing is known about the chemokine pathways that promote homing of T reg cells to allografts.

We now provide evidence that Foxp3 expression is detected within cardiac allografts and that expression is markedly enhanced by therapies that promote tolerance induction. In addition, we show that naturally occurring T reg cells provide intragraft Foxp3 expression, and that their homing to allografts is both CCR4 dependent and required for allograft tolerance.

RESULTS AND DISCUSSION
### High Foxp3 expression after CD154 mAb therapy

We undertook serial analysis of Foxp3 levels in a standard murine vascularized allograft model across a full MHC mismatch. Splenic Foxp3 mRNA levels decreased progressively post-Tx but increased in cardiac allografts (Fig. 1 a). Increases in graft Foxp3 were not simply responses to surgery or ischemia/reperfusion injury as levels in isografts were far less than allografts (Fig. 1 b). However, allograft Foxp3 levels in untreated recipients at 7 d post-Tx were dwarfed compared with grafts associated with CD154 mAb/DST therapy (Fig. 1 b). Expression for >60 d after Tx was also shown by Northern analysis (Fig. 1 c). Foxp3 protein was localized to a subset of infiltrating mononuclear cells in allografts of mice treated with CD154 mAb/DST (Fig. 1 d). These data indicate that, whereas Foxp3$^+$ cells traffick to sites of active immunity, far higher levels are seen at the same sites and persist long-term in allograft recipients treated with CD154-directed costimulation blockade.

Presence of intragraft Foxp3$^+$ cells varies according to the therapeutic protocol

We evaluated whether Foxp3 expression was a common feature in long-surviving allografts, regardless of the therapeutic protocol. Recipients treated with CD154/DST had permanent allograft survival (>100 d) and accepted second donor-strain cardiac allografts without any additional therapy (Fig. 2 a), but rejected normally third-party donor allografts (not depicted). We noted previously that treatment of CD28$^{-/-}$ recipients with anti–inducible costimulator (ICOS) mAb also led to permanent engraftment (>100 d; reference 16), and now found that such long-term recipients did not show evidence of donor-specific tolerance when challenged with a second donor-strain cardiac allograft (Fig. 2 a). The presence of CD25$^+$ cells was essential for tolerance induction using CD154/DST for the following reasons: (a) thymectomy plus CD25 mAb therapy depleted resting CD4$^+$CD25$^+$ T reg cells and prevented long-term allograft survival and (b) depletion of the same cell population in recipients bearing well-functioning cardiac allografts after CD154/DST therapy restored allograft rejection (Fig. 2 a, inset).
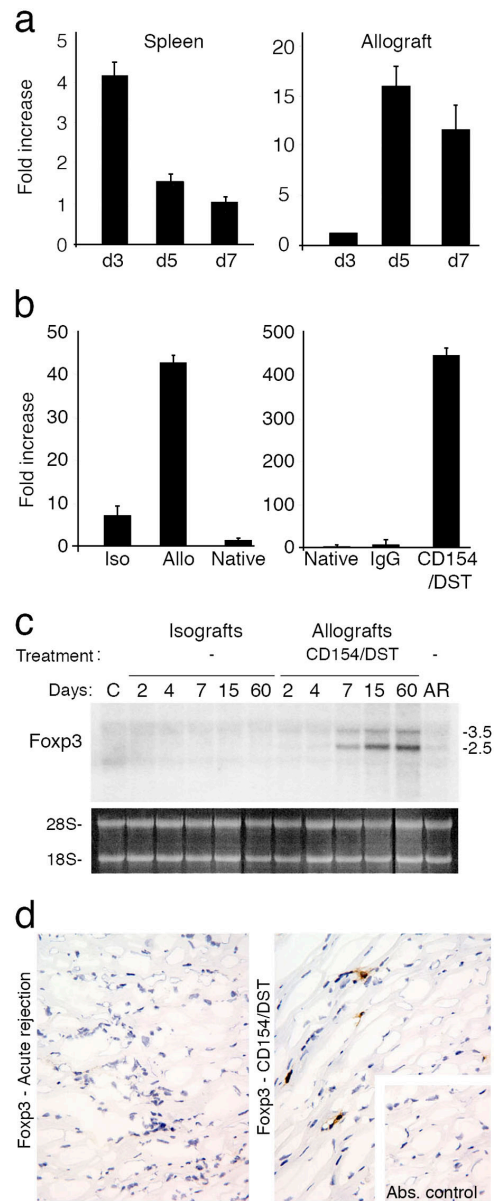
Figure 1.   Post–Tx Foxp3 expression. (a) qPCR analysis showed a progressive decrease in splenic Foxp3 mRNA (left, mean ± SD) and an increase in associated cardiac grafts (right); expression shown relative to levels at 7 or 3 d post-Tx, respectively. (b) Comparison at 7 d post-Tx showed increases in allograft Foxp3 mRNA (qPCR, mean ± SD) were >5-fold that of C57BL/6 isografts (left), whereas levels in grafts from recipients treated with CD154 mAb/DST therapy were >100-fold higher than control grafts (right); expression in each panel is relative to native heart. (c) Confirmation of intragraft Foxp3 mRNA expression by Northern analysis, comparing expression in control (C) normal hearts, acutely rejecting (day 7) allografts or serially harvested isografts versus allografts from hosts treated with CD154 mAb/DST as indicated. (d) Foxp3 staining was exceedingly rare in allografts undergoing acute rejection (left), but readily detected as Foxp3$^+$ mononuclear cells in mice treated with CD154 mAb/DST; immunoperoxidase labeling of hematoxylin-stained cryostat sections. Original magnification, ×250 (inset shows lack of staining with Foxp3 peptide-absorbed antibody).
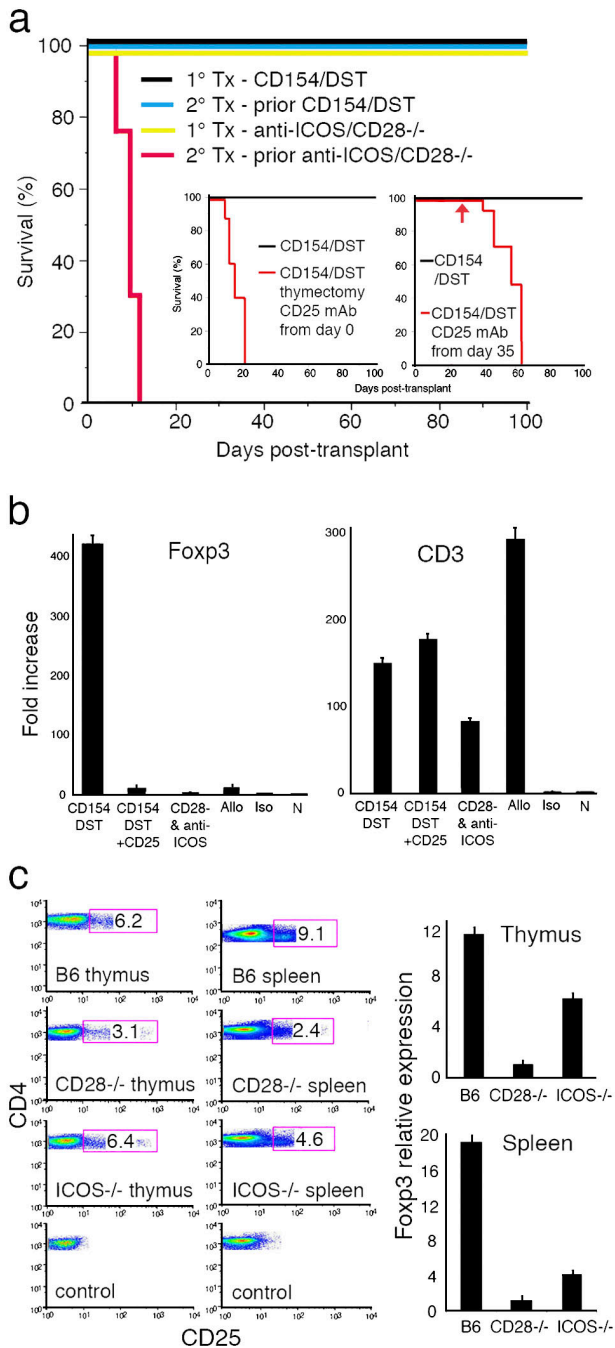
Figure 2. Foxp3, T reg cell, and costimulation blockade. (a) CD154/DST induced long-term cardiac allograft survival and donor-specific tolerance, whereas CD28$^{-/-}$ recipients treated with neutralizing anti-ICOS mAb also accepted primary cardiac allografts long term but rejected second donor allografts. Inset shows how thymectomy and CD25 mAb therapy prevents long-term allograft survival despite CD154/DST therapy (left), and that CD25^{+} cells are also required for maintenance of allograft survival (right) because CD25 mAb therapy beginning at day 35 restores allograft rejection. (b) Levels of Foxp3 and CD3 mRNA (qPCR, mean ± SD) in day 7 cardiac allografts. (c) Flow cytometry showing decreased CD4^{+}CD25^{+} cells in thymic and splenic samples of control, CD28$^{-/-}$ or ICOS$^{-/-}$ mice; numbers are percentage of CD4^{+}CD25^{+} cells within gates set using an

These data suggested the utility of comparing two protocols that induced permanent allograft survival but differed in their ability to induce allograft tolerance. qPCR analysis of cardiac allografts showed that high levels of Foxp3 mRNA expression were restricted to mice receiving the tolerizing CD154/DST protocol (Fig. 2 b). The large differences in intragraft Foxp3 expression were not due to differences in leukocyte infiltration as levels of CD3 transcripts in rejecting allografts versus those destined for long-term allograft survival varied only two- to threefold (Fig. 2 b). Moreover, long-surviving (>100 d) donor-strain cardiac allografts, but not acutely rejected third-party cardiac allografts, in mice whose first allografts were performed under CD154 mAb/DST coverage also showed high intragraft Foxp3 expression (unpublished data).

Previous studies demonstrated that CD28 costimulation is important for optimal development and maintenance of T reg cells in thymus and periphery (17), and that ICOS blockade impairs the functions of Tr1-type T reg cells (9). Consistent with this, naive CD28$^{-/-}$ mice had <30% of the thymic and splenic CD4^{+}CD25^{+} cells of wild-type controls, CD4^{+}CD25^{+} cells were decreased by >50% in ICOS$^{-/-}$ mice compared with controls, and qPCR showed markedly decreased expression of Foxp3 in CD28$^{-/-}$ and ICOS$^{-/-}$ mice (Fig. 2 c). Hence, decreased intragraft expression of Foxp3 in CD28$^{-/-}$ recipients treated with anti-ICOS mAb at least partially reflects impaired production of Foxp3^{+} T reg cells. We do not know yet if therapy with CTL antigen-4–Ig, which interrupts CD28–B7 interactions and when used in conjunction with DST can promote long-term allograft survival, results in alterations in intragraft Foxp3 expression.

Foxp3 expression post–Tx does not correlate with T reg cell markers or cytokine expression

Comparison by qPCR at 7 d post-Tx showed allografts in untreated recipients had the highest levels of CD25 and GITR expression (Fig. 3 a). Although both CD25 and GITR are used as markers of T reg cells, their expression is increased upon T cell activation, rendering them of limited use post-Tx. Indeed, elevated levels of intragraft GITR in untreated recipients are consistent with a role for the GITR–GITR ligand interaction in reversing the suppressive activity of T reg cells and providing positive costimulatory signals to responder T cells (18).

We also sought an association between intragraft mRNA levels of key cytokines and Foxp3 expression. Although no association with IL-2, IFN–γ, or IL-4 was apparent (unpublished data), the TGF-β and IL-10 findings were of interest given recent literature. The relation of T reg cells that constitutively express Foxp3, so-called naive T reg cells, to T reg cells that arise in the periphery, "induced" T reg cells, is unclear. TGF-β

CD25 isotype mAb. (d) Analysis of corresponding Foxp3 mRNA (qPCR) with expression shown relative to levels in samples from CD28$^{-/-}$ mice; results are representative of three independent experiments.
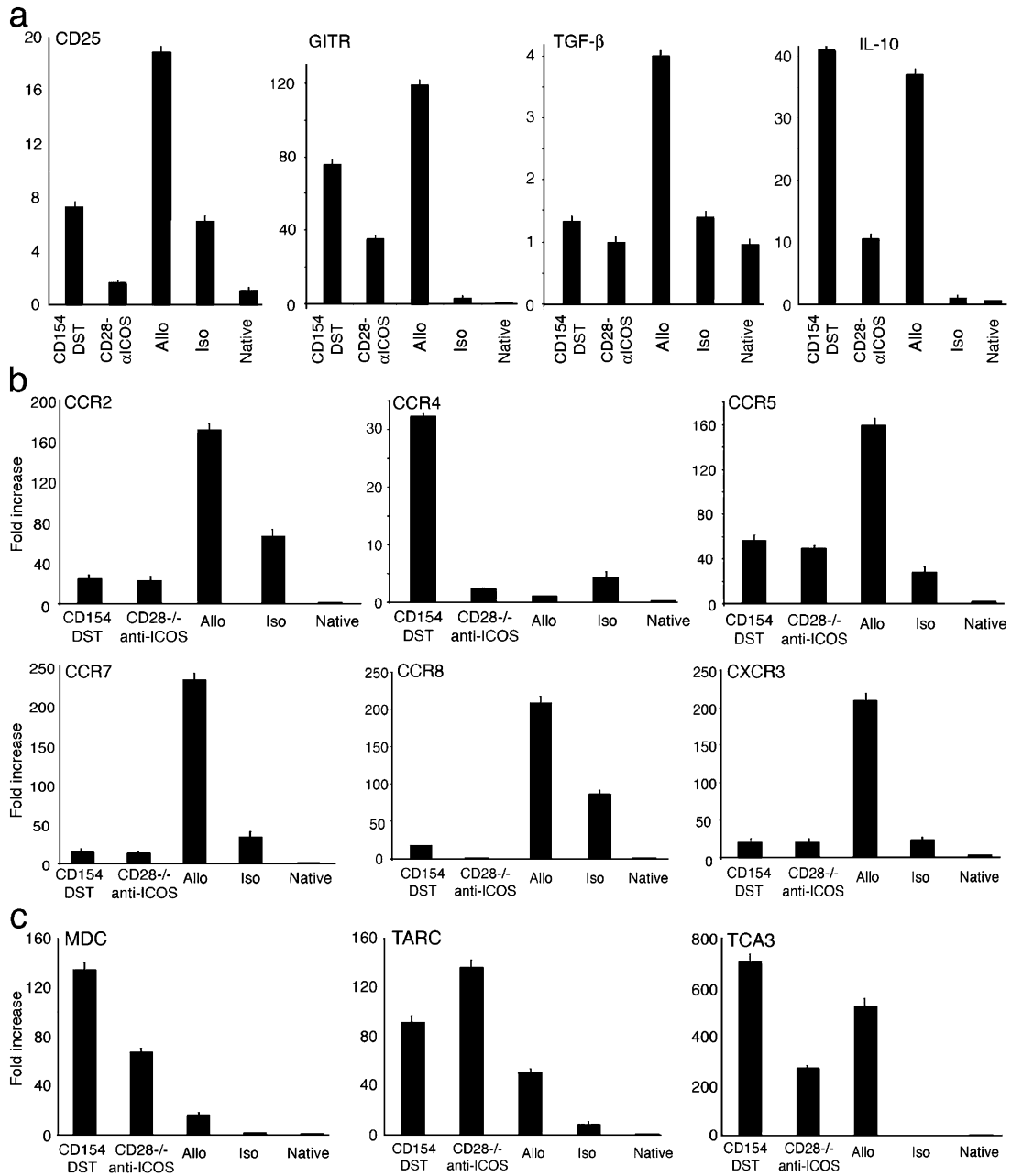
Figure 3. Intragraft mRNA expression of immunoregulatory molecules and chemokine pathways at day 7 post–Tx. (a) Intragraft levels of CD25, GITR, TGF-β, and IL-10 did not correlate with selective high Foxp3 expression in CD154 mAb/DST grafts. (b) Comparison of intragraft chemokine receptor expression. (c) Intragraft expression of CCR4 (MDC, TARC) and CCR8 (TCA3) ligands. qPCR results, which are shown relative to expression in native hearts, are representative of three independent experiments.

promotes conversion of naive T cells to T reg cells via induction of Foxp3 but does not promote expansion of murine naive T reg cells in vitro (19, 20) and, in contrast with induced T reg cells, naive T reg cells require cell contact to exert inhibitory effects (19, 21). Our qPCR analysis failed to show a correlation between Foxp3 (Fig. 2 b) and TGF-β mRNA intragraft expression (Fig. 3 a), and though IL-10 levels were markedly elevated in tolerized recipients, they were also high

in allografts of untreated mice (Fig. 3 a). High levels of TGF-β and IL-10 in allografts harvested at 7 d from untreated recipients likely reflect the intense inflammatory response occurring at this point, resulting in graft destruction. Analyses at later time points are clearly required to assess the nature of intragraft cytokine expression and its relationship to tolerance induction. However, at least 7 d post–Tx, the current data involving mRNA profiling, allograft survival, and effects of CD25+ cell

depletion are most consistent with expansion of a subset of Foxp3^{+}, likely naive T reg cells that do not necessarily appear to require TGF-β or IL-10 for growth or function.
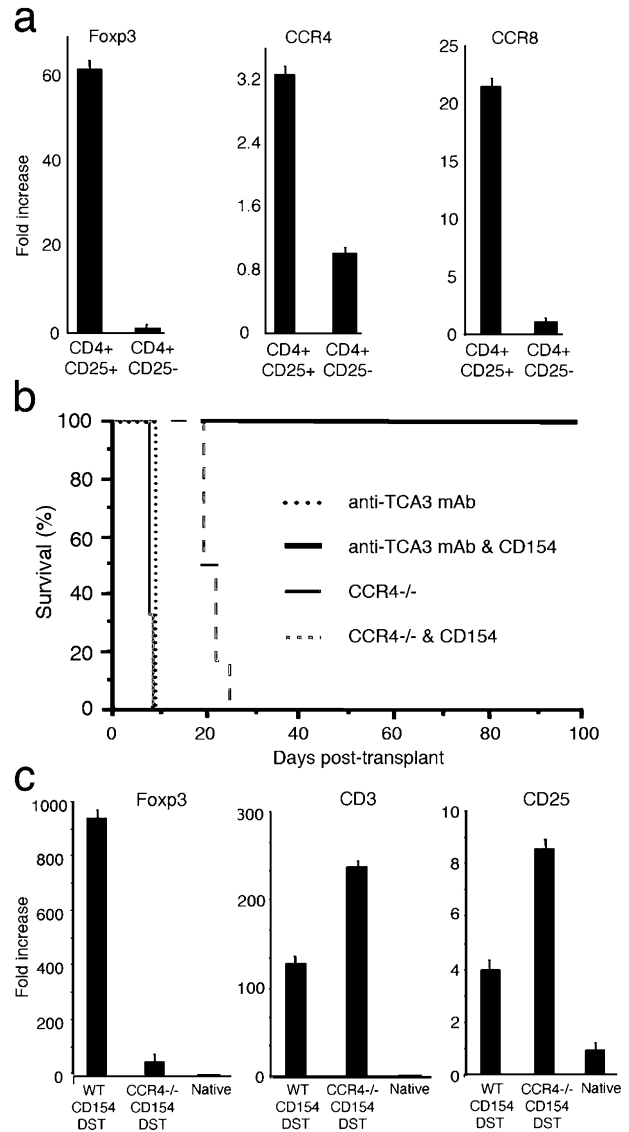
Foxp3[+] T reg cell recruitment and tolerance induction is CCR4 dependent

Previous studies in this model have shown that specific chemokine/chemokine receptor pathways recruit host effector cells to allografts and are key to allograft rejection (22). Therefore, we assessed whether any chemokine receptors were linked with homing of T reg cells post-Tx. As anticipated (22), expression of the chemokine receptors, CCR2, CCR5, and CXCR3, were highest in rejecting allografts, and their levels in allografts destined for long-term survival were equivalent to, or below, levels in isografts (Fig. 3 b), but two exceptions to this pattern were apparent.

First, the chemokine receptor, CCR4, was preferentially expressed in allografts from CD154/DST–treated recipients (Fig. 3 b) that expressed the most Foxp3. Moreover, serial analysis of recipient spleens by qPCR on days 3, 5, and 7 post-Tx suggested that, like Foxp3^{+} cells, CCR4^{+} cells leave the spleen and migrate to the graft (unpublished data). There was also increased expression of one of the two main CCR4 ligands, macrophage-derived chemokine (CCL22), but not thymus- and activation-regulated chemokine (TARC; CCL17), in CD154 mAb/DST-treated grafts (Fig. 3 c). The basis for this relatively selective expression of one ligand over the other is unknown. However, after submission of our paper, a study of patients with ovarian carcinoma was published that showed that host responses to the tumor cells were impaired by local Foxp3^{+} CCR4^{+} T reg cells that were recruited as a result of tumor production of MDC and not TARC (23).

Second, and less strikingly, although levels of CCR8 were far higher in rejecting allografts than those harvested from mice receiving either costimulation blockade protocol, comparison of just the latter showed a relative preponderance of expression of CCR8 (Fig. 3 b) and its ligand, TCA3 (Fig. 3 c) in recipients treated with CD154 mAb/DST. Among various receptors, human CD4^{+}CD25^{+} T reg cells are known to express CCR4 and CCR8 and display chemotactic responses to their ligands, such as MDC and I-309 (the human TCA3 homologue), respectively (24), leading us to further consider the potential importance of the CCR4 and CCR8 pathways in our model.

Comparison of purified CD4^{+}CD25^{+} and CD4^{+}CD25^{−} T cells by qPCR confirmed expression of Foxp3, CCR4, and CCR8 by CD4^{+}CD25^{+} cells (Fig. 4 a), and expression of CCR4 by T reg cells was markedly enhanced upon CD3/CD28 mAb-induced activation (not depicted). Neutralization of TCA3 had no effect on the tempo of allograft rejection or on the ability of CD154 mAb/DST to promote long-term graft survival (Fig. 4 b). In contrast, although CCR4^{−/−} allograft recipients rejected their grafts at the normal rate, CCR4^{−/−} recipients failed to show prolonged graft

Figure 4. **Key role of CCR4 in T reg cell recruitment and tolerance induction.** (a) qPCR analysis of Foxp3, CCR4, and CCR8 mRNA expression by CD4^{+}CD25^{+} versus CD4^{+}CD25^{−} T cells; cells were isolated using magnetic beads to >95% purity. Data are expressed as relative to levels in CD4^{+}CD25^{−} cells and are representative of three separate experiments. (b) Targeting of TCA3 using a neutralizing mAb had no effect on allograft rejection in wild-type recipients or long-term allograft survival when administered in conjunction with CD154 mAb/DST therapy. In contrast, CCR^{−/−} recipients rejected allograft normally and could not be tolerized using CD154 mAb/DST therapy. (c) Analysis by qPCR of intragraft levels of Foxp3, CD3, and CD25 mRNA expression at day 7 post-Tx, showing marked reduction in intragraft Foxp3 but not CD3 or CD25 in CCR4^{−/−} versus control mice treated with CD154 mAb/DST; results are shown relative to expression in native hearts and are representative of three independent experiments.

survival despite CD154 mAb/DST therapy (Fig. 4 b). Allografts harvested from CCR4^{−/−} recipients at 7 d post-Tx had markedly decreased expression of Foxp3^{+} mRNA (Fig. 4 c), despite levels of T cell infiltration reflected in CD3
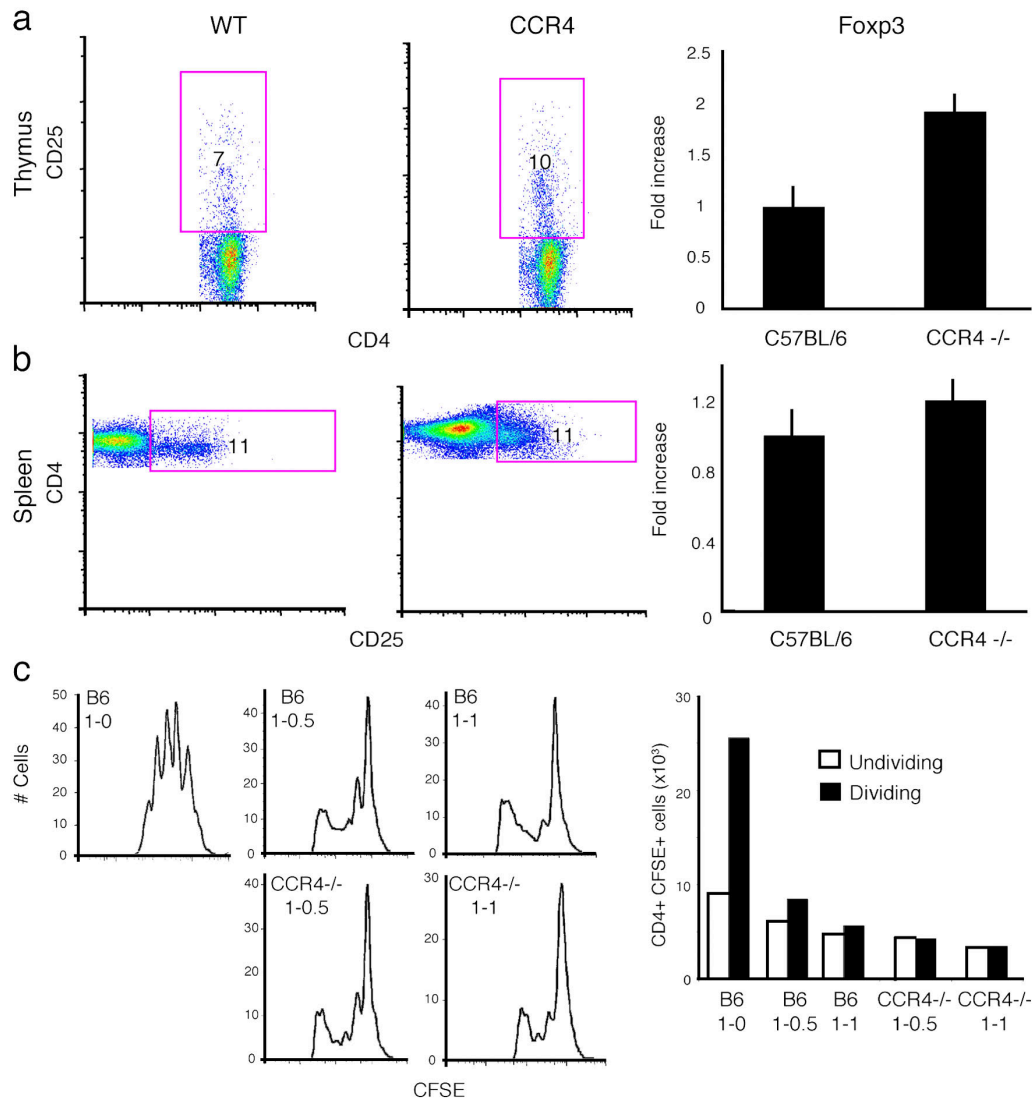
Figure 5. Numbers and function of T reg cells in CCR4$^{-/-}$ mice are comparable to WT controls. (a) FACS analysis of CD25$^+$ expression by CD4$^+$ cells from naive thymus, and corresponding qPCR analysis of Foxp3 mRNA expression in whole thymus from WT and CCR4$^{-/-}$ mice. (b) FACS analysis of CD25$^+$ expression by CD4$^+$ cells in naive spleen and corresponding qPCR analysis of Foxp3 mRNA expression in whole spleen. (c) CFSE-labeled CD4$^+$CD25$^-$ responder T cells exhibited decreased CD3 mAb-induced CFSE proliferation in the presence of CD4$^+$CD25$^+$ T reg cells from either WT or CCR4$^{-/-}$ mice at the indicated ratios, as compared with no T reg cells added. (right) Histograms show the results of quantitation of the absolute number of responder CD4$^+$CFSE$^+$ cells in the absence and presence of T reg cells from WT and CCR4$^{-/-}$ mice.

transcripts, which were broadly comparable between rejecting allografts in CCR4$^{-/-}$ mice and tolerized grafts in control mice. Associated analysis of CD25 expression demonstrated its limited utility as a marker of T reg cells within the first week post-Tx because levels of CD25 mRNA were somewhat enhanced in CCR4$^{-/-}$ recipients, consistent with expression by activated effector T cells mediating acute allograft rejection.

Failure of tolerance induction in CCR4$^{-/-}$ mice was unlikely to be due to defects in the immune repertoire of these animals as we found the numbers and function of T reg cells in CCR4$^{-/-}$ mice were comparable to that of wild-type mice (Fig. 5, a–c). Furthermore, CCR4$^{-/-}$ mice displayed intact Th1 immunity, rejecting cardiac allograft similarly to controls, and are known to develop normal Th2-type immune responses (25).

In summary, our data provide new insights into the mechanisms of tolerance induction after costimulation blockade. Strategies involving CD154 but not CD28/ICOS costimulation blockade induced donor-specific tolerance in association with trafficking into the graft of a population of CD4$^+$CD25$^+$ T reg cell–expressing Foxp3. This trafficking to the allograft is also required for maintenance of allograft tolerance. Lastly, expression profiles and

mechanistic studies indicate that migration of T reg cells to cardiac allografts after costimulation blockade with CD154/DST in this model is uniquely dependent upon the CCR4 chemokine receptor pathway.

MATERIALS AND METHODS

Surgery. We performed heterotopic vascularized cardiac allografting (15) using 6–8 wk BALB/c (H-2^d) donors and wild-type, CD28$^{-/-}$ (26), ICOS$^{-/-}$ (16), or CCR4$^{-/-}$ (25) C57BL/6 (H-2^b) recipients (n = 4–6 grafts/group). Rejection, defined as cessation of ventricular contractions, was confirmed by histology. C57BL/6 mice (4–6 wk) were thymectomized as described previously (27). Mice were housed in specific pathogen-free conditions and studied using a protocol approved by the Institutional Animal Care and Committee of the Children's Hospital of Philadelphia.

Flow cytometry. T cells from thymic and splenic samples were analyzed using CD4-FITC and CD25-PE (BD Biosciences) mAb conjugates or isotype controls (28). CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells were isolated from splenic samples (>95% purity) by negative selection using the magnetic cell separation system (MACS), using the manufacturer's recommendations (Miltenyi Biotec).

Therapies. Allografted mice received CD154 mAb (MR1, Bio-Express, 250 μg) plus i.v. DST (5 × 10^6 cells; reference 15), control hamster IgG, or DST alone at surgery; anti-ICOS mAb (12A8, 200 μg, i.p.; reference 16); or control rat IgG every other day for 14 d. The contribution of CD25$^+$ cells to tolerance was assessed with a depleting CD25 mAb (PC61): (a) thymectomized mice were treated with CD25 mAb (250 μg, i.p., two doses); (b) allograft recipients were treated with CD25 mAb on days 0, 2, and 4 post-Tx; or (c) allograft recipients with well-functioning grafts after CD154/DST therapy were treated at 35 and 37 d post-Tx. Efficacy of CD25$^+$ cell depletion was monitored by flow cytometry using a second CD25 mAb (7D4). We neutralized the CCR8 ligand, TCA3 (CCL1), with a hamster mAb (B12D6; reference 29), using 200 μg daily i.v. for 14 d post-Tx.

RNA studies. For quantitative PCR analysis (qPCR), RNA extraction, reverse transcription, cDNA amplification using specific primer and probe sequences for target genes (TaqMan PDAR; Applied Biosystems), and standardization were performed as described previously (28). Full-length 1,312-bp Foxp3 cDNA was amplified from total thymus RNA using forward 5′-GAACCCAATGCCCAACCCTAG-3′ and reverse 5′-TTCTTGGT-TTTGAGGTCAAGGG-3′ primers, and used for Northern analysis (16).

Immunopathology. Allograft histology was assessed in hematoxylin and eosin– and elastin-stained paraffin sections, and infiltrating cells were analyzed by immunoperoxidase using rat anti–mouse mAbs (16). Foxp3 was detected with an affinity-purified rabbit antibody generated against a 13-mer peptide TFPRSGTPRKDSN corresponding to amino acids 169–181 of mouse Foxp3; a single band of ∼52 kD was seen by Western blotting of mouse thymocytes.

T cell proliferation assays. CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells plus APCs were isolated from spleen and lymph node samples by negative selection (MACS; reference 28); 5 × 10^4 responder CD4$^+$CD25$^-$ cells were labeled with carboxyfluorescein succinimidyl ester (CFSE), cocultured with equal numbers of APCs in the presence of 0.5 μg/ml CD3 with or without CD4$^+$CD25$^+$ T reg cells at the indicated ratios, and CFSE proliferation was assessed 48 h later.

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1044

TOLERANCE AND CCR4-DEPENDENT RECRUITMENT OF FOXP3$^+$ T REG CELLS | Lee et al.