Different Mechanisms Control Peripheral and Central Tolerance in Hematopoietic Chimeric Mice


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Regulatory T cells (Treg) are important in peripheral tolerance, but their role in establishing and maintaining hematopoietic mixed chimerism and generating central tolerance is unclear. We now show that costimulation blockade using a donor-specific transfusion and anti-CD154 antibody applied to mice given bone marrow and simultaneously transplanted with skin allografts leads to hematopoietic chimerism and permanent skin allograft survival. Chimeric mice bearing intact skin allografts fail to generate effector/memory T cells against allogeneic targets as shown by the absence of IFNγ-produing CD4hiCD8− T cells and in vivo cytotoxicity. Depletion of Tregs by injection of anti-CD4 or anti-CD25 antibody prior to costimulation blockade prevents chimerism, shortens skin allograft survival, and leads to generation of effector/memory cytotoxic T cells. Depletion of Tregs by injection of anti-CD4 or anti-CD25 antibody two months after transplantation leads to loss of skin allografts even though mice remain chimeric and exhibit little in vivo cytotoxicity. In contrast, chimerism is lost, but skin allografts survive following naive T-cell injection. We conclude that hematopoietic chimerism and peripheral tolerance may be maintained by different mechanisms in mixed hematopoietic chimeras.

Key words: Anti-CD154 mAb, chimerism, costimulation blockade, regulatory T cell

Abbreviations: DST, donor-specific transfusion; GVHD, graft versus host disease; mAb, monoclonal antibody; MHC, major histocompatibility complex; MST, median survival time; PBMCs, peripheral blood mononuclear cells; Treg, regulatory T cell.

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Introduction

Bone marrow transplantation protocols based on costimulation blockade and requiring minimal or no myeloablative conditioning have been described in mice (1–14). Regulatory T cells (Tregs) are important mediators of peripheral tolerance in many systems (15–24), but their role in induction and maintenance of hematopoietic chimerism and transplantation tolerance in mice treated with costimulation blockade remains unclear. Reports have variously suggested that Tregs are not required for either the induction or maintenance of chimerism (25) or are important only for the induction but not maintenance of chimerism (7,14). One report suggested that CD4+CD25+ Tregs are important for the induction but not the maintenance of chimerism (14).

In studies of peripheral tolerance induction, we have shown that permanent islet and prolonged skin allograft survival can be achieved in mice treated with a donor-specific transfusion (DST) plus anti-CD154 monoclonal antibody (mAb) (26,27). Skin allograft survival is shortened by injection of anti-CD4 (27,28) or anti-CD25 mAb (29), and CD4+CD25+ and CD4+CD25− Tregs play important roles in allograft survival depending on the tissue graft transplanted (29). We have recently shown that this peripheral tolerance induction protocol also permits the establishment of hematopoietic chimerism and generation of central tolerance if bone marrow is transplanted during costimulation...
blockade and donor-specific skin allografts are transplanted 8 weeks later (30).

In the present study, we first document that simultaneous transplantation of bone marrow and skin allografts leads to long term stable chimerism and permanent skin allograft survival. We next show that Tregs are important in the induction of hematopoietic chimerism. Finally, we show that depletion of Tregs in stable mixed hematopoietic chimeras leads to the loss of skin allografts but not chimerism, whereas injection of naïve T cells leads to loss of chimerism but not skin allografts. These data suggest that chimerism and peripheral tolerance are maintained by two different mechanisms in mixed hematopoietic chimeras.

Materials and Methods

Animals
BALB/cJ (H2d), C57BL/6J (H2b), C3H/HeJ (H2k), and CBA/J (H2k) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in microisolator cages, given ad libitum access to autoclaved food, and maintained in accordance with the guidelines of Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Antibodies and flow cytometry
Purified anti-mouse CD154 mAb was obtained from The National Cell Culture Center (Minneapolis, MN) (29,31). Anti-CD4 mAb, FITC-conjugated anti-H-2-Kd mAb, PE-conjugated anti-H-2-Kb, APC-conjugated anti-CD4 mAb, FITC-conjugated anti-CD25 mAb, PerCPC- conjugated anti-CD8 mAb, PE-Cy7-conjugated anti-IFNγ mAb, PE-conjugated anti-CD19, PE-conjugated CD11b, APC-conjugated anti-Ly6G Ly6C, FITC-conjugated CD11c, APC-conjugated anti-CD11b mAb, and corresponding isotype control antibodies were purchased from BD PharMingen (San Diego, CA). The anti-CD122 mAb was generated from the TM-ß1 hybridoma (anti-mouse IL-2 receptor ß chain, rat IgG2b (32), the gift of T. Tanaka, Osaka University Medical School).

IFNγ-producing CD8+ T cells were detected with the CytoTox/Cytoperm Kit Plus with GolgiPlug (BD Pharmingen) as previously described (33). Briefly, cells were incubated with C57BL/6 (H2b), BALB/c (H2d) or CBA/J (H2k) stimulator spleen cells in the presence of human rIL-2 (BD PharMingen) and GolgiPlug for 5 hr at 37°C. After incubation, cells were stained for cell surface markers, fixed and permeabilized with Cytofix/Cytoperm solution, and stained with anti-IFNγ mAb. Labeled cells were analyzed with a FACScan instrument (Becton Dickinson, Sunnyvale, CA), and ~10 × 10^6 events were acquired for each analysis.

Donor-specific transfusion and mAb treatment
BALB/c male mice 6–8 weeks of age were treated with a DST consisting of 10 × 10^6 allogeneic C57BL/6 spleen cells injected intravenously. DST was given on day −7, and 0.5 mg of anti-CD154 mAb was injected intraperitoneally on days −7, −4, 0, and +4 relative to C57BL/6 bone marrow and skin transplantation on day 0. Anti-CD4 mAb (OKT1, 0.5mg) or anti-CD25 mAb (PC61, 0.25mg) was injected intraperitoneally relative to transplantation at the time points indicated in the text and legends. We confirmed ~99% CD4+ and ~95% CD25+ T cell depletion by flow cytometry using antibodies against noncompeting CD4 (RM4–4) and CD25 (7D4) epitopes.

Simultaneous bone marrow and skin transplantation
Single cell suspensions of C57BL/6 bone marrow were prepared from femurs and tibias, filtered through 70 μm nylon mesh (Becton Dickinson, Franklin Lakes, NJ), washed and counted. BALB/c mice received a single intravenous injection of 50 × 10^6 bone marrow cells (30). Full-thickness C57BL/6 skin grafts 1–2 cm in diameter were transplanted onto the dorsal flanks of recipients (30). Skin graft survival was evaluated three times a week, and graft rejection was defined as the first day on which the entire graft was necrotic.

Determination of chimerism
The percentage of peripheral blood mononuclear cells (PBMCs) expressing major histocompatibility complex (MHC) class I in chimeric mice was determined by labeling with antibodies to H2-Kd (C57BL/6, donor) and H2-Kb (BALB/c, host) (30). Because fewer than 100% of cells express MHC class I antigen, the relative percentage of donor-origin cells in chimeric recipients was calculated as follows: [% donor cells/[% donor cells+[% host cells]) × 100 (30). Based on staining using isotype controls, we defined chimeric recipients as mice in which the percentage of donor-origin cells in PBMC was >0.10% at 8 weeks.

Adoptive transfer
Hematopoietic chimeric BALB/c mice bearing intact C57BL/6 skin allografts 35 weeks after simultaneous bone marrow and skin transplantation were injected intravenously with 50 × 10^6 naïve BALB/c spleen cells.

In vivo cytotoxicity assay
The in vivo cytotoxicity assay was performed as previously described (34). Briefly, splenocytes from C57BL/6 (H2b, allogeneic), BALB/c (H2d, syngeneic), and C3H/HeJ (H2b, third-party) donors were labeled with different concentrations of CFSE (2.5, 0.74, and 0.22 μM), washed, and mixed at equal ratios; 1 × 10^7 cells of each population were injected intravenously into recipients. Twenty hours later, recipient spleen cells were recovered and analyzed by flow cytometry for the presence of each population based on relative CFSE intensity. NK cells were depleted 1–2 days prior to adoptive transfer of CFSE-labeled cells by an intraperitoneal injection of 1 mg of anti-CD122 mAb (35) to permit quantification of alloreactive CD8+ T-cell cytotoxicity in the absence of NK cell killing. Specific lysis was calculated as described (34).

Statistical methods
The average duration of graft survival is presented as the median. Duration of graft survival among groups was compared by life-table analysis using the method of Kaplan and Meier; the equality of survival distributions in different treatment groups was tested using the log rank statistic (36). Comparisons of three or more means used one-way analyses of variance and Bonferroni adjusted unpaired t-tests (37) for a posteriori contrasts (GraphPad Software, Version 4.0, San Diego, CA). Comparisons of two means used unpaired t-tests without assuming equal variance (37). Analyses of 2 × 2 tables used Fisher’s exact test (37). The p values <0.05 were considered statistically significant.

Results
Costimulation blockade in mice simultaneously given bone marrow and skin allografts establishes multilineage mixed hematopoietic chimerism and leads to permanent skin allograft survival
We have shown that mice treated with a DST, anti-CD154 mAb, and transplanted with allogeneic bone marrow develop hematopoietic chimerism and central tolerance (30). In those experiments, skin allografts were transplanted 8 weeks after bone marrow transplantation to allow central tolerance to develop prior to skin grafting. To investigate whether bone marrow and skin allografts could be
transplanted together, BALB/c mice were treated with a C57BL/6 DST plus anti-CD154 mAb and simultaneously given C57BL/6 bone marrow and skin allografts.

Eight weeks after transplantation, donor hematopoietic cells were detected in most mice (Table 1, group 1). In a cohort of chimeric mice with intact skin allografts (N = 4) analyzed at 8 weeks that exhibited donor hematopoietic chimerism levels of 5.8 ± 2.6% in the blood, 8.1 ± 1.0% in the spleen and 7.0 ± 1.2% in the bone marrow, multilineage hematopoietic cell engraftment was observed (Figure 1). CD4:CD8 CD3+ T-cell ratios were (3.6:1) in the blood and (1.8:1) spleen.

In contrast, mice given costimulation blockade and skin allografts but no bone marrow did not develop chimerism (<0.10%, Table 1, group 2). The median survival time (MST) of the skin allografts on these nonchimeric mice was prolonged, but all grafts eventually failed (MST = 49 days, Figure 2, panel A).

In mice chimeric at 8 weeks, the MST of skin allografts in was >245 days (Figure 2, panel A), although not all skin allografts survived to the end of the experiment. In the six mice that were nonchimeric at 8 weeks (<0.1% donor), skin allograft survival was short and all were eventually rejected (MST = 39, range 8–109 days).

To determine if skin allograft rejection was associated with loss of chimerism, hematopoietic tissues of seven mice that were chimeric 8 weeks after transplantation were studied at the end of the observation period. Four of these mice had intact grafts whereas three had rejected their skin allografts on days 81, 104 and 143. Chimerism (1.8–6.8%) was detected in all lymphoid tissues studied in the four mice with intact skin allografts (Table 2, group 1a) whereas only minimal chimerism was observed in mice that had rejected their skin allografts (Table 2, group 1b).

To document that tolerance established in chimeric mice was donor-specific, the four BALB/c (H2b) mice bearing intact C57BL/6 (H2d) skin grafts were then transplanted ~35 weeks after transplantation with third-party C3H/HeJ (H2k) skin allografts. Although C3H/HeJ grafts were rapidly rejected (MST = 11 days, N = 4, range 10–13 days), the original C57BL/6 skin allografts remained intact until the experiment was terminated 5 weeks later (p < 0.01 vs. third-party allografts).

**Anti-CD4 mAb or anti-CD25 mAb treatment prior to costimulation blockade prevents establishment of hematopoietic chimerism and shortens skin allograft survival**

Injection of anti-CD4 mAb or anti-CD25 mAb prior to costimulation blockade blocks peripheral tolerance induction and shortens skin allograft survival (29), but its effect on bone marrow cell engraftment is not clear. To investigate this, BALB/c mice were depleted of CD4+ or CD25+ cells prior to costimulation blockade and transplantation with C57BL/6 bone marrow and skin allografts.

The majority of mice not injected with anti-CD4 or anti-CD25 mAb became chimeric (Table 1, group 3) and skin allograft survival was prolonged. Skin allografts remained intact in 11 of 13 chimeric mice to the end of the experiment (MST > 155 days, Figure 2, panel A). In contrast, only 2 of 23 mice treated with anti-CD4 mAb became chimeric (Table 1, group 4) and all 23 mice rapidly rejected their skin allografts (MST = 18 days, Figure 2, panel A). Six of 19 mice treated with anti-CD25 mAb generated low levels of chimerism at 8 weeks that was decreased to near background levels at 18 weeks (Table 1, group 5). Skin allograft survival in this group was brief (MST = 21 days, Figure 2, panel A).

**Treatment with anti-CD4 mAb or anti-CD25 mAb prior to costimulation blockade leads to the generation of effector/memory CD8+ T cells**

We next asked if anti-CD4 or anti-CD25 mAb treatment prevents the establishment of hematopoietic chimerism.
Figure 1: Multilineage chimerism is established in mixed chimeras. BALB/c mice were treated with a C57BL/6 DST on day −7 and four doses of 0.5 mg anti-CD154 mAb on days −7, −4, 0, and +4 relative to transplantation with C57BL/6 skin and 50 × 10⁶ C57BL/6 bone marrow cells on day 0. At 8 weeks, blood (panel A), spleen (panel B), and bone marrow (panel C) were recovered from mice bearing intact skin allografts and analyzed by flow cytometry for percent donor hematopoietic chimerism and the presence of multilineage donor hematopoietic cells. The percentages of CD3, CD19, CD11b⁺/Ly-6G,6C⁺, CD11b⁺/Ly-6G,6C⁻, CD11c⁺/Gr1⁺, and CD11c⁺/Gr1⁻, and CD11c⁺/CD11b⁻ cells represent their proportion of donor (H2-Kb) cells that express these phenotypes. Data represent mean ± SD of four mice.

by permitting generation of effector/memory T cells. To investigate this, we used an in vivo cytotoxicity assay for detection of effector/memory allogeneic CD8⁺ T cells (34).

In mice given costimulation blockade, in vivo cytotoxic activity against donor-specific allogeneic targets on the day of bone marrow transplantation (day 0) was low (Figure 3, panel A), documenting that costimulation blockade prevents the generation of effector/memory CD8⁺ T cells. In mice given costimulation blockade and injected with anti-CD4 mAb or anti-CD25 mAb, in vivo cytotoxic activity was high against allogeneic C57BL/6 (H2b) splenocytes but not against third-party C3H/HeJ splenocytes (Figure 3, panel A).

In similar experiments, an in vivo cytotoxicity assay was performed 30 weeks after transplantation using mice from the same three treatment groups described in Figure 3, panel A. Mice treated with costimulation blockade and given bone marrow cells were chimeric, retained intact skin allografts, and continued to exhibit low in vivo cytotoxic activity (Figure 3, panel B). In contrast, mice injected with either anti-CD4 or anti-CD25 mAb prior to costimulation blockade exhibited low levels of chimerism, had rejected their skin allografts, and exhibited high in vivo cytotoxicity activity against donor-specific but not third-party allogeneic targets (Figure 3, panel B).

To investigate further the effects of anti-CD4 or anti-CD25 mAb on alloreactive T cells, we adapted an intracellular cytokine assay for detecting effector/memory virus-specific CD8⁺ T cells (38) for use in identifying alloreactive T cells (39). In this assay, IFNγ production is a marker of effector/memory CD8⁺ T cells (31,33,38,39).

As expected, costimulation blockade prevented the generation of effector/memory IFNγ-producing T cells (Figure 4, panel A). In contrast to the in vivo cytotoxicity assay, no effector/memory IFNγ-producing T cells could be detected in mice also treated with anti-CD4 or anti-CD25 mAb (Figure 4, panel A). These data suggest that the in vivo cytotoxicity assay is a more sensitive indicator of effector/memory cell generation than the intracellular cytokine assay.

In similar experiments, an intracellular IFNγ cytokine assay was performed 30 weeks after transplantation using mice from the same three treatment groups described in Figure 4, panel A. Chimeric mice with intact skin allografts evidenced only low levels of IFNγ-producing CD44⁺CD8⁺ T cells (Figure 4, panel B). In contrast, high levels of IFNγ-producing CD44⁺CD8⁺ (effector/memory) splenic T cells were present in mice that had been injected prior to costimulation blockade with anti-CD4 mAb or anti-CD25 mAb and had also rejected their skin allografts, (Figure 4, panel B).

Anti-CD4 mAb or anti-CD25 mAb treatment of hematopoietic chimeric mice with intact skin allografts leads to rejection of the skin allograft but not the hematopoietic graft

We next asked whether Tregs were required to maintain chimerism and skin allografts. Chimeric BALB/c mice with intact skin allografts (Table 3) were injected with anti-CD4 mAb or anti-CD25 mAb and had also rejected their skin allografts (Figure 4, panel A).
Figure 2: Skin allograft survival on mice treated with costimulation blockade and transplanted with bone marrow cells and skin allografts with or without prior administration of anti-CD4 or anti-CD25 mAb. Panel A: BALB/c mice were treated with a C57BL/6 DST on day −7 and four doses of 0.5 mg anti-CD154 mAb on days −7, −4, 0 and +4 relative to skin transplantation with or without injection of $50 \times 10^6$ C57BL/6 bone marrow cells on day 0. In separate cohorts, BALB/c mice were also treated with a depleting anti-CD4 mAb (0.5 mg) on days −10, −9 and −8 or anti-CD25 mAb (0.25 mg) on day −10 relative to skin and bone marrow transplantation. Shown is the skin allograft survival on mice chimeric at 8 weeks in Table 1. There were no significant differences in the duration of skin allograft survival in groups 1 and 3 in Table 1, and these have been pooled for presentation. *p < 0.0001 vs. all other groups. Vertical bars indicate censored data. Panel B: BALB/c mice were treated with a C57BL/6 DST on day −7 and four doses of 0.5 mg anti-CD154 mAb on days −7, −4, 0 and +4 relative to skin transplantation and injection of $50 \times 10^6$ C57BL/6 bone marrow cells on day 0. Mice with intact skin allografts at day 57 relative to skin transplantation were divided into three groups. One group received no further treatment. One group received anti-CD4 mAb (0.5 mg) on days +57, +58 and +59. One group received anti-CD25 mAb (0.25 mg) on day +57. Shown is the skin allograft survival of mice in Table 1. *p < 0.001 vs. all other groups. No other differences between groups were statistically significant.
mAb 57–59 days after transplantation. Skin graft rejection occurred in 19 of 20 mice (MST = 59 days after mAb treatment, Figure 2, panel B). Similarly, injection of anti-CD25 mAb 57 days after transplantation led to rejection of 13 of 20 skin allografts (MST = 75 days after mAb treatment, Figure 2, panel B). Although skin allografts were rejected, all anti-CD4 or anti-CD25 mAb-treated mice maintained a stable chimerism 10 through 28 weeks (2–20 weeks after mAb treatment) after bone marrow transplantation that was comparable to that observed in mice not treated with anti-CD4 or anti-CD25 mAb (Table 3 and Figure 5, panel A).

To further investigate these findings, we performed in vivo cytotoxicity assays on these three cohorts of mice 30 weeks after transplantation. As expected, chimeric mice bearing intact skin allografts exhibited no donor-specific cytotoxic activity (Figure 5, panel B). Anti-CD4 mAb injected mice that had rejected their skin allografts but remained chimeric exhibited only low levels of allogeneic cytotoxicity. Anti-CD25 mAb-injected mice that had rejected their skin allografts but remained chimeric exhibited undetectable levels of cytotoxicity (Figure 5).

**Hematopoietic chimeras require Treg cells for maintenance of skin allograft survival but not hematopoietic chimerism**

Rejection of skin allografts after anti-CD4 or anti-CD25 mAb suggests that Tregs are required for the maintenance of skin allograft survival but not for hematopoietic chimerism. To confirm the importance of Tregs for maintaining skin allografts in chimeric mice, four chimeric BALB/c mice bearing intact C57BL/6 skin allografts were injected with 50 × 10⁶ naïve BALB/c spleen cells 35 weeks after bone marrow and skin transplantation. Within 4 weeks of adoptive transfer, hematopoietic chimerism in these mice decreased to nondetectable levels (<0.1%), but despite the loss of chimerism, only one of four skin allografts was eventually rejected (on day 55 after cell transfer).

**Discussion**

Protocols that establish allogeneic hematopoietic chimerism without the need for myeloablation or chronic immunosuppression would permit much more widespread implementation of transplantation therapy in the clinic. We (30) and others (6,8–10,13) have developed costimulation blockade protocols in mice that achieve this goal, but prior to adapting these protocols for clinical use, it is important to understand the underlying mechanisms.

In the present study, we document that simultaneous transplantation of bone marrow and skin allografts to mice treated with costimulation blockade establishes hematopoietic chimerism and leads to the permanent survival of skin allografts. Costimulation blockade prevents the generation of effector/memory CD8 cytotoxic T cells. Depletion of CD4+ or CD25+ cells prior to the initiation of costimulation blockade prevents establishment of chimerism, leads to the generation of alloreactive effector/memory cells, and results in rapid skin allograft rejection. Maintenance of chimerism does not appear to depend on Tregs. In contrast, even in hematopoietic chimeras, survival of skin allografts is Treg-dependent.

We have previously shown that DST plus anti-CD154 mAb permits bone marrow engraftment, generation of central tolerance, and permanent survival of skin allografts transplanted 8 weeks later (30). We now extend this observation by documenting that simultaneous transplantation of bone marrow and skin, prior to the establishment of central tolerance, also leads to permanent skin allograft survival.

Interestingly, some chimeric mice lost their chimerism by 8 weeks and subsequently rejected their skin allografts. One possible explanation is that our costimulation blockade protocol induces tolerance in the alloreactive T-cell compartment, but not in the alloreactive NK cell compartment. It has recently been shown that, even in mice that have been treated with costimulation blockade to depress alloreactive T-cell activity, potent NK cell activity persists and leads to
In vivo cytotoxic activity in mice treated with anti-CD4 mAb or anti-CD25 mAb at the time of transplantation and 30 weeks after transplantation. BALB/c mice were randomized into three groups. All mice were treated with C57BL/6 DST on day −7 and two doses of 0.5 mg of anti-CD154 mAb on days −7 and −4 relative to analysis on day 0. One group was given no additional mAb treatment. One group was injected with three doses of 0.5 mg of anti-CD4 mAb on days −37, −39 and −42. One group was injected with 0.25 mg of anti-CD25 mAb on day −18. All mice were injected with anti-CD122 mAb to deplete NK cells 24–48 h prior to transfer of CFSE-labeled syngeneic (BALB/c) and donor-specific (C57BL/6) or third party (C3H/HeJ) allogeneic spleen cell targets. Panel A: Mean percentages of cytotoxic activity in mice analyzed on day 0 (the day of transplantation) as compared to mice not treated with anti-CD4 or anti-CD25 mAb are shown. *p < 0.001 vs. cytotoxic activity in no-antibody treated recipients of C57BL/6 allogeneic cells. Panel B: Mean percentages of cytotoxic activity in mice 30 weeks after transplantation from the same three treatment groups described in panel A. At this time, all mice treated with costimulation blockade and given bone marrow had intact skin allografts whereas all mice injected with either anti-CD4 mAb or anti-CD25 mAb at the time of costimulation blockade had rejected their skin allografts. *p < 0.001 vs. cytotoxic activity in no-antibody treated recipients of C57BL/6 allogeneic cells.

rejection of allogeneic hematopoietic grafts (40). This observation is in agreement with our previous report that, in mice treated with DST and anti-CD154 mAb, NK cell depletion facilitated hematopoietic cell engraftment, and establishment of chimerism (30).

To understand how peripheral tolerance induction facilitates establishment of hematopoietic chimerism and transplantation tolerance, we used an in vivo cytotoxicity assay to show that our peripheral tolerance induction protocol prevents the generation of effector/memory CD8+ T cells.
We speculate that alloreactive T-cell deletion permits immediate survival of skin allografts and that establishment of hematopoietic chimerism leads to the generation of central tolerance and long-term skin allograft survival. Because long-term chimeras with intact skin allografts fail to generate cytotoxic alloreactive T cells, these data suggest that hematopoietic chimerism leads to deletional tolerance, an interpretation consistent with previous reports showing...
that mixed chimerism leads to deletional tolerance (5,41–44).

The role of Tregs in establishing and maintaining chimerism is not clear. To investigate this issue, we used anti-CD4 and anti-CD25 mAbs to delete Tregs. One study has reported no role for Treg activity in either establishing or maintaining chimerism (25), but chimerism levels were much higher in that report. Two other reports demonstrated that injection of anti-CD4 at the time of costimulation blockade prevents establishment of chimerism (7,14). Interestingly, the potent Treg population in one study was CD4+CD25+, and this Treg population was not required 2 weeks after transplantation for the maintenance of chimerism. In a study in which chimerism was induced in C57BL/6 mice treated with rapamycin, anti-CD154 mAb, CTLA4Ig, and 200 in which chimerism was induced in C57BL/6 mice treated for the maintenance of chimerism. In a study this Treg population was not required 2 weeks after transplantation after costimulation blockade had already been initiated. In the present study, CD4 and CD25 cells were depleted prior to tolerance induction. Overall, our data suggest that CD25+ Tregs are important for induction of hematopoietic chimerism and for permanent skin allograft survival in chimeric mice.

In long-term chimeras, injection of either anti-CD4 or anti-CD25 mAb led to loss of skin allografts despite persistence of hematopoietic chimerism. This suggests that hematopoietic chimerism in our system may be maintained by deletional tolerance as previously suggested by others (5,41–44). Our present results are also in agreement with a previous report showing that Tregs do not play a major role in maintaining chimerism (25). However, even in chimeric mice with intact skin allografts, injection of either antibody results in graft rejection without loss of hematopoietic chimerism, perhaps due to deletion of Tregs present in the graft as previously suggested (41). Furthermore, injection of naive splenocytes led to the loss of hematopoietic chimerism but not skin allograft rejection. The requirement for Tregs for maintenance of skin allografts but not hematopoietic chimerism may be due to lower levels of chimerism achieved in our study than in previous reports that showed clonal deletion was the primary mechanism for skin allograft survival (5,42–44). If the antigenic specificities expressed by donor skin and marrow are similar, then our observation that Tregs are required to maintain skin allografts suggests that deletion of donor-reactive T cells cannot be complete. However, the level of deletion obtained, albeit incomplete, must be sufficient to maintain chimerism but not skin allografts. An alternative explanation for our observations is that Tregs are required to maintain tolerance to skin-specific antigens that are not present on hematopoietic grafts.

We have previously reported different costimulatory requirements for induction of tolerance to skin vs. islet allografts (45) and different requirements for Tregs in maintenance of skin versus islet allografts (29). The present data extend this differential requirement for Tregs to that of skin allografts and hematopoietic chimerism. Our results contrast with those of another study in which injection of naive splenocytes into chimeric mice failed to shorten skin

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All mice were treated with a donor-specific transfusion (DST) on day −7 and four injections of 0.5 mg of anti-CD154 mAb on days −7, −4, 0 and +4 relative to simultaneous bone marrow and skin allograft transplantation on day 0. Group 2 mice were also injected with three doses of 0.5 mg of anti-CD4 mAb on days 57, 58, and 59, and group 3 mice were also injected with 0.25 mg of anti-CD25 mAb on day 57. Hematopoietic chimerism was defined as >0.10% donor-origin PBMCs 8 weeks after bone marrow injection. All mice in each group were chimeric when tested at 8 weeks after bone marrow cell transplantation prior to the time of anti-CD4 or anti-CD25 mAb injection. Data are mean ± 1 SD.

Using our protocol, treatment with anti-CD4 or anti-CD25 mAb prior to the initiation of costimulation blockade leads to rapid skin allograft rejection (29). Anti-CD4 but not anti-CD25 mAb prevents the deletion of high affinity TCR transgenic alloreactive KB5 CD8+ T cells in our ‘synchronic’ model system in which we can determine the fate of alloreactive CD8+ T cells (29). In the present study, we now show that treatment with either antibody prevents the establishment of hematopoietic chimerism and leads to potent in vivo cytotoxic activity. We have previously shown that anti-CD4 mAb given simultaneously with costimulation blockade or anti-CD25 mAb given 6 days after costimulation blockade but before bone marrow transplantation do not block development of chimerism (30). Two possible reasons could account for the different results obtained in the present study. Simultaneous skin allografting could result in increased antigen load, rendering the mice more sensitive to lack of Treg activity. Alternatively, the timing of CD4 depletion between the two studies may be important. In our previous study, anti-CD4 mAb treatment and initiation of costimulation blockade was done simultaneously, and anti-CD25 mAb treatment was done immediately prior to transplantation after costimulation blockade had already
Tregs, Chimerism and Allograft Survival

Figure 5: Chimerism and in vivo cytotoxicity of mice treated with anti-CD4 mAb or anti-CD25 mAb. BALB/c mice were treated with a C57BL/6 DST on day −7 and four doses of 0.5 mg anti-CD154 mAb on days −7, −4, 0 and +4 relative to skin transplantation and injection of $50 \times 10^6$ C57BL/6 bone marrow cells on day 0. Mice with intact skin allografts at day 57 relative to skin transplantation were divided into three groups. One group received no further treatment. One group received anti-CD4 mAb (0.5 mg) on days +57, +58, and +59. One group received anti-CD25 mAb (0.25 mg) on day +57. Panel A: Shown is the level of chimerism in the blood of the mice shown in Figure 2b and Table 3 when analyzed 28 weeks after transplantation. At this time, all mice treated with costimulation blockade and given bone marrow cells had intact skin allografts whereas all mice treated with costimulation blockade, given bone marrow and injected with either anti-CD4 mAb or anti-CD25 mAb had rejected their skin allografts. There were no significant differences in the frequency of chimeric mice in any of the groups or the level of chimerism in the blood at this time point ($p = NS$). Each point represents an individual mouse. Horizontal bars represent the median of each group. Panel B: Mice from the same three treatment groups described in panel A were entered into an in vivo cytotoxic assay 30 weeks after transplantation. CFSE-labeled donor-specific and allogeneic cells were injected into mice treated with anti-CD122 mAb 24–48 hours earlier. Mean percentages of cytotoxic activity to donor-specific C57BL/6 (H2b) and third-party C3H/HeJ (H2k) splenocytes are shown. * $p < 0.01$ vs. cytotoxic activity in mice not treated with anti-CD4 or anti-CD25 mAb.

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allograft survival or lead to loss of hematopoietic chimera (41). These differences may be due to the tolerance induction protocols used or the different hosts used in the studies (C57BL/6 vs. BALB/c). The differences may also be due to the fact that in our system, both major and minor MHC differences exist whereas in the B10.A into C57BL/6 system used in the other study, only major, not minor MHC differences were encountered (41).

In summary, hematopoietic chimera in mixed chimeras appears to be maintained by a deletional mechanism whereas skin allograft survival requires Tregs. Methodology to enhance Treg activity in combination with costimulation blockade could lead to the elimination of myeloablative conditioning as a requirement for establishing hematopoietic chimera in the clinic.

References

9. Kean LS, Durham MM, Adams AB et al. A cure for murine sickle cell disease through stable mixed chimera and tolerance induction after nonmyeloablative conditioning and major histocompati-

American Journal of Transplantation 2007; 7: 1710–1721


