Combinations of Anti-LFA-1, Everolimus, Anti-CD40 Ligand, and Allogeneic Bone Marrow Induce Central Transplantation Tolerance through Hemopoietic Chimerism, Including Protection from Chronic Heart Allograft Rejection

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Central transplantation tolerance through hemopoietic chimerism initially requires inhibition of allogeneic stem cell or bone marrow (BM) rejection, as previously achieved in murine models by combinations of T cell costimulation blockade. We have evaluated LFA-1 blockade as part of regimens to support mixed hemopoietic chimerism development upon fully allogeneic BALB/c BM transfer to nonirradiated busulfan-treated B6 recipient mice. Combining anti-LFA-1 with anti-CD40 ligand (CD40L) induced high incidences and levels of stable multilineage hemopoietic chimerism comparable to chimerism achieved with anti-CD40L and everolimus (40-O-(2-hydroxyethyl)-rapamycin) under conditions where neither Ab alone was effective. The combination of anti-LFA-1 with everolimus also resulted in high levels of chimerism, albeit with a lower incidence of stability. Inhibition of acute allograft rejection critically depended on chimerism stability, even if maintained at very low levels around 1%, as was the case for some recipients without busulfan conditioning. Chimerism stability correlated with a significant donor BM-dependent loss of host-derived Vβ11+ T cells 3 mo after BM transplantation (Tx). Combinations of anti-CD40L with anti-LFA-1 or everolimus also prevented acute rejection of skin allografts transplanted before established chimerism, albeit not independently of allospecific BMTx. All skin and heart allografts transplanted to stable chimeras 3 and 5 mo after BMTx, respectively, were protected from acute rejection. Moreover, this included prevention of heart allograft vascular intimal thickening (“chronic rejection”). The Journal of Immunology, 2004, 173: 7025–7036.

Bone marrow (BM)1 or hemopoietic stem cell transplantation (Tx) often constitutes part of a therapy for human hemopoietic deficiencies and malignancies (1, 2). Notwithstanding its proven clinical potential for subsequent allograft tolerance (3), toxicity of conditioning regimens and complications arising from alloreactivity have thus far precluded the clinical use of BMTx for tolerance induction (4). Studies in animal models, therefore, have aimed to minimize conditioning and to optimize immune modulation for allogeneic BMTx (5). These have included allospecific immune modulation instead of general host T cell depletion 3 mo after BMTx, and it was necessary and sufficient to prevent acute skin allograft as well as acute and chronic heart allograft rejection.

Materials and Methods

Mice

Female 6- to 8-wk-old BALB/c, C57/B16 (B6), and C3H mice were obtained from Charles River Breeding Laboratories (L’Arbresle, France). Mice were housed under conventional conditions in filter-top-protected cages and cared for in accordance with Swiss federal law.

Preparation of BM and reagents

BM was flushed from tibiae, femurs, and humeri. Viable white blood cells within unfractionated BM cell suspensions were adjusted to 20 × 10⁶ per 0.5 ml of i.v. injection.

Abbreviations used in this paper: BM, bone marrow; CD40L, CD40 ligand; Tx, transplantation.
Hamster anti-mouse CD40L (CD40L, CD154; clone MR1) and rat anti-mouse LFA-1 (clone M17) were purified by the core facilities (Novartis Pharma, Basel, Switzerland). Purified hamster Ig and rat Ig (ChromPure: Jackson ImmunoResearch Laboratories, West Grove, PA) were purchased from Dianova (Hamburg, Germany). Abs were injected i.p. in 200 μl of PBS. Everolimus (Novartis Pharma) was diluted in 20% cremophor/ethanol/water/80% PBS and injected i.p. at 3 mg/kg in 200 μl. Busulfan (Sigma-Aldrich, Buchs, St. Gallen, Switzerland) was dissolved in 20% DMSO/80% of equal parts of polyethylene glycol in water and administered 1 day before BMTx.

### Flow cytometry

Hemopoietic chimerism in peripheral blood was analyzed by coexpression of H-2Dd (biotinylated, clone 34-2-12, with streptavidin-PE) and CD3 (clone 145-2C11, FITC conjugate) with CD4 (clone RM4-5, allophycocyanin conjugate) or CD8 (clone 53-6-7, PerCP conjugate). B220 (clone RA3-6B2, PerCP conjugate) was used as a B cell marker and CD11b (clone M17/0, FITC conjugate) for monocytes/granulocytes. Vβ11+ and Vβ8+ TCR-positive T cells were analyzed with anti-CD3-PerCP, anti-Vβ8-PE (clone MR5-2), anti-Vβ11-FITC (clone RR3-15), and anti-H-2Dd-biotin followed by streptavidin-allophycocyanin. For cell fixation and RBC lysis, samples were treated with CAL-Lyse (Caltag Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Data were acquired and analyzed on a FACS Calibur (BD Biosciences, San Jose, CA) using CellQuest software. The percentage of chimerism for a given cell type is the percentage of H-2Dd+ -positive cells among PBL gated for the relevant marker.

### Results

B6 mice received a single dose of 2 × 10^7 BALB/c BM cells 1 day after busulfan administration. For most experiments, mice were preconditioned with a single dose of 30 mg/kg busulfan, previously shown to induce high levels of mixed chimerism with syngeneic BMtx or allogeneic BMtxs and costimulation blockade (13). Anti-CD40L, anti-LFA-1, and everolimus were dosed as shown in Fig. 1 and combined as indicated for individual figures and tables. Heart Tx was performed in chimeric mice that had received BM and skin grafts ~5 and 2 mo before, respectively (Fig. 1A). Alternatively, BM and skin were transplanted within a 2-day interval, followed by secondary skin grafts 3 mo later (Fig. 1B).

**Synergies among anti-LFA-1, anti-CD40L, and everolimus for the development of hemopoietic chimerism**

Blocking Abs against LFA-1 and CD40L and everolimus were tested for their ability to support fully allogeneicmatched hemopoietic chimerism (Fig. 2). The kinetics of chimerism development and chimerism levels were compared for each treatment (Fig. 2, rows a–f) and for the granulocyte/monocyte, B cell, and T cell lineages (columns from left to right: CD11b, B220, CD4 T cells, and CD8 T cells). BM recipients treated with either anti-CD40L, anti-LFA-1, or everolimus alone failed to develop chimerism for any of these lineages (Fig. 2, a–c), whereas combinations of anti-CD40L with everolimus or anti-LFA-1 induced high levels of stable chimerism for all lineages and with comparable kinetics (Fig. 2, d and e). The combination of anti-LFA-1 plus everolimus resulted in similar levels and kinetics of stable chimerism among all lineages (Fig. 2f), albeit at lower incidences; in this experiment one of four compared with three of four after anti-CD40L plus everolimus (Fig. 2d) and four of four after anti-LFA-1 plus anti-CD40L (Fig. 2e). As discussed below, however, this was not the most representative outcome for the anti-LFA-1 plus everolimus combination which, on average over many experiments, resulted in ~50% stable chimerism, and 50% slow loss of chimerism in all lineages.

**Relationship between chimerism stability and host-allospecific T cell depletion**

A reproducible observation from several experiments was chimerism instability in some anti-LFA-1 plus everolimus-treated BM recipients, whereas chimerism after anti-LFA-1 plus anti-CD40L treatment was always stable (Fig. 3A). To address possible mechanisms and consequences of chimerism loss in this system, we tested allospecific host T cell depletion and transplantation tolerance.

For the BALB/c to B6 combination, donor- dependent host T cell depletion can be monitored by the reduction of host Vβ11+ or Vβ8+ TCR T cells specific for donor I-E-restricted endogenous retroviral superantigens (10, 11, 13, 28). In this study, we measured host Vβ11+ and Vβ8+ TCR chain expression to represent T cells that would or would not undergo deletion under the influence of donor BM, respectively. In chimeric BM recipients, host-type (gated H-2Dd-negative) Vβ11+ T cells declined over time from 5–6% to 1–2% while Vβ8+ T cells showed a small concomitant and probably compensatory increase from 16 to 18% to 19 to 22%. The Vβ8/Vβ11 ratio was used to indicate these changes (Fig. 3A). Although there was no direct positive correlation between chimerism levels and Vβ8/Vβ11 ratios, a comparison between stable and
unstable chimerism revealed a clear correlation between a significant increase of the Vß8:Vß11 ratio (resulting from a reduction of Vß11+ T cells) at 3 mo after BMTx and chimerism stability (Fig. 3A, p = 0.002): three of six BM recipients pretreated with anti-LFA-1 plus everolimus displayed sharply declining granulocyte/monocyte chimerism between 1 and 3 mo after BMTx (Fig. 3Ab, open symbols). One of these BM recipients (Fig. 3Ab, open triangles) without measurable Vß11 depletion completely lost chimerism by 3 mo. Two others (Fig. 3, open circle and open square) showed moderate transient increases to 20% in B and T cell chimerism by 2 mo and a decline to 10% by 3 mo. Moderate T cell chimerism of only 10% by 3 mo was also detected for another BM recipient, albeit in combination with high B cell and granulocyte/monocyte chimerism of ~80% (Fig. 3Ab, open symbol). Similarly increased Vß8:Vß11 ratios of 7–9 were measured at 3 mo for two of three BM recipients with only 10% T cell chimerism (Fig. 3Ab, open circle and crossed symbol), both of which were still chimeric for all lineages 8 mo after BMTx, yet at very different levels of ~90%/80%/8% and 10%/8%/8% chimerism for monocytes, B cells, and T cells, respectively. By contrast, for another animal with 10% T cell chimerism at 3 mo (Fig. 3Ab, open squares), the Vß8:Vß11 ratio was barely increased above basal levels at this time and chimerism was lost 8 mo after BMTx. These data suggest, therefore, that the degree of Vß11 depletion 3 mo after BMTx may be more predictive for subsequent chimerism stability than chimerism levels per se.

Chimerism status and skin graft acceptance

Inhibition of skin allograft rejection is a routine readout for transplantation tolerance in mice. For the present study, the same animals shown in Fig. 3A were used to test the role of hemopoietic chimerism and chimerism stability in this system (Fig. 3B). Skin Tx was performed 3 mo after BMTx. BM recipients that failed to develop chimerism lost both BALB/c and third-party C3H skin grafts through early acute rejection around days 7–11. One anti-LFA-1 plus everolimus-treated BM recipient that had lost chimerism by the time of skin Tx rejected skin grafts as early as recipients that had never displayed detectable chimerism (Fig. 3, A and Bb, open triangles). When chimerism was significant at the time of skin Tx and subsequently lost over a longer period of time, this delayed but did not prevent acute skin graft rejection (Fig. 3, Ab and Bb, open squares). The delay of acute rejection to day 16 specifically correlated with previous transient chimerism, because the same animal acutely rejected its third-party C3H skin graft by day 9. Another BM recipient of early declining but subsequently low level stable chimerism never showed any signs of acute rejection of the BALB/c skin graft, whereas third-party C3H skin was acutely rejected by day 9 (Fig. 3, Ab and Bb, open circles). Together, these data suggest that the stability of chimerism is critical for allograft tolerance in this model, regardless of the combination treatment used to achieve chimerism and without apparent correlations between tolerance and chimerism levels.

Although stable hemopoietic chimeras never lost BALB/c skin grafts through acute rejection, most grafts eventually changed through a slow process of increasing dryness and hair loss in the absence of overall inflammation and necrosis similar to previously reported chronic rejection through skin-specific Ags (29–32). These changes were specific for BALB/c skin grafts, because syngeneic B6 skin grafts cotransplanted with BALB/c skin onto some chimeric recipients remained completely intact until the end of the experiment.
Loss of chimerism correlates with enhanced host anti-donor alloreactivity

Allograft tolerance through hematopoietic chimerism was shown to be reflected by loss of in vitro alloreactivity in MLR assays (32). Lack of MLR activity was confirmed with spleens from chimeric skin graft recipients (Fig. 4). As expected, positive MLR was detected with spleens from BM recipients that failed to develop chimerism. The strength of this MLR was comparable for responder spleens from naive B6 mice (Fig. 4). More vigorous in vitro alloreactivity, however, was consistently obtained with responder spleens from BM recipients that had slowly lost previously significant levels of chimerism (Fig. 4). Different MLR kinetics were excluded from this interpretation, as spleen cells from all animals responded with the same kinetics to irradiated BALB/c target cells (Fig. 4, inset). Enhanced in vitro alloreactivity by BM recipients with slow loss of chimerism argues against loss of chimerism as an immunologically neutral event and presumably reflects previous in vivo alloreactivity against donor-type hematopoietic cells.

Short-term combination treatments of anti-CD40L with anti-LFA-1 or everolimus protect concomitant skin and BM grafts, with ensuing central tolerance induction, but they do not induce peripheral tolerance

Combination treatments of anti-CD40L plus anti-LFA-1 and anti-CD40L plus everolimus appeared to be equally effective for hematopoietic chimerism development and skin allograft tolerance. With tighter timing of BMTx and skin Tx however, strategies of immune modulation would have to prevent both donor-specific BM and skin graft rejection before the establishment of central tolerance.
tolerance. Such a protocol would be closer to some clinical situations of concurrent cadaveric BM and organ grafting, and it might reveal different efficacies between combination treatments as well as a potential for BM-independent allograft protection. To address these issues, mice received BM and skin grafts within 2 days while applying the same short-term treatment regimens of anti-CD40L with anti-LFA-1 or everolimus as outlined in Fig. 1B. Three months later, all mice received secondary BALB/c and third-party C3H skin grafts. Allogeneic BMTx resulted in stable chimerism and specific inhibition of acute skin allograft rejection in all recipients and with both combinations (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngeneic BMTx controls, both combination treatments delayed but did not prevent acute rejection of primary skin grafts (Tables I and II, group 1). For syngen...
suggested memory responses to BM-independent skin Ag(s), because stable multilineage chimerism was maintained in all cases.

**Allogeneic BM does not induce alloantibody production, even in the absence of stable chimerism**

Our previous results were consistent with a mechanism of transplantation tolerance that was strictly dependent on central T cell tolerance through stable hemopoietic chimerism. By contrast, transient chimerism in nonhuman primates was sometimes sufficient to support long-term renal allograft survival (33). It was proposed for these cases that the organ graft itself might be involved in the maintenance of peripheral tolerance. Another suggested mechanism was the induction of B cell tolerance by BMTx, thereby avoiding alloantibody-related complications. The latter possibility was supported by a report showing that chances for renal allograft survival in the context of BMTx were greatly increased by splenectomy. Other monkeys developed IgG alloantibodies after kidney Tx and rejected their grafts after the loss of chimerism (34). In murine systems, chimerism-induced tolerance has been more extensively studied for cellular than for humoral immunity. For our system, we asked how alloantibodies might be related to the failure of stable chimerism and skin graft rejection. B6 recipients of BALB/c BM were treated with anti-LFA-1 plus everolimus with or without BALB/c skin Tx 2 days after BMTx. IgM and IgG alloantibodies were detected by flow cytometry of BALB/c PBL T cells stained with B6 plasma prepared 8–9 mo after BMTx (Table III). Complete failure of chimerism in a control group treated with everolimus alone did not result in alloantibody production (Table III, group 2). Among nine anti-LFA-1 plus everolimus-treated recipients of BALB/c BMTx with stable chimerism (no skin Tx), eight mice did not develop IgM or IgG alloantibodies and only one recipient was positive for allogeneic BM but not IgG (Table III, group 3). One animal from this group displayed the characteristic pattern of declining multilineage chimerism that was lost by 3 mo after BMTx. Alloreactive IgM or IgG was not detected however, suggesting that loss of chimerism in this model was not accompanied by alloantibody formation (Table III, group 3). Remarkably, still no alloantibodies were detectable when an anti-LFA-1 plus everolimus-treated recipient of both BALB/c BM and skin slowly lost its chimerism over 3 mo and also lost its skin graft through delayed acute rejection 46 days after Tx (Table III, group 4). This lack of alloantibodies appeared to depend on previously detected chimerism, because one animal from the same treatment group that completely failed to develop chimerism and acutely rejected its skin graft with a moderate delay on day 18 clearly contained allospecific IgG (group 4, mean fluorescence intensity

Table I. **Skin allograft tolerance with anti-CD40L and anti-LFA-1 treatment depends on allospecific BMTx**

| Treatment | Skin Graft1 BALB/c | Skin Graft1 C3H | Skin Graft2 BALB/c | Skin Graft2 C3H | Stable Chimerism |
|-----------|-------------------|-----------------|-------------------|----------------|-----------------|
| **Group 1** |                   |                 |                   |                 |                 |
| Anti-CD40L, anti-LFA-1, BALB/c BM | >day 270 | acute, median day = 35 | >day 1170 | acute, median day = 9 | 7/7 |
|            | >day 270 |                 | >day 170 |                 |                 |
|            | >day 270 |                 | >day 170 |                 |                 |
|            | Chronic from day 89 |                 | Acute day 15 |                 |                 |
|            | Chronic from day 91 |                 | Chronic from day 15 |             |                 |
|            | Chronic from day 67 |                 | Acute day 17 |             |                 |
| **Group 2** |                   |                 |                   |                 |                 |
| Anti-CD40L, anti-LFA-1, B6 BM | acute, median day = 36 | acute, median day = 9 | Acute, median day = 9 | 0/7 |
| **Group 3** |                   |                 |                   |                 |                 |
| Hamster Ig, rat Ig, B6 BM | acute, median day = 12 | acute, median day = 12 | acute, median day = 10 | 0/7 |

*Protocols were applied as outlined in Fig. 1B.*
Table II. Skin allograft tolerance with anti-CD40L and everolimus depends on allospecific BMTx

| Treatment | Skin Graft1 BALB/c | Skin Graft1 C3H | Skin Graft2 BALB/c | Skin Graft2 C3H | Stable Chimerism |
|-----------|-------------------|-----------------|--------------------|----------------|-----------------|
| Group 1   | Anti-CD40L, everolimus, BALB/c BM | >day 275 | Acute, median day = 18 | >day 175 | Acute, median day = 9 | 7/7 |
|           | Anti-CD40L, everolimus, BALB/c BM | >day 126 | Chronic from day 50 | Chronic from day 156 | Acute day 10 |
| Group 2   | Anti-CD40L, everolimus, B6 BM | Acute, median day = 17 | Acute, median day = 17 | Acute, median day = 10 | Acute, median day = 8 | 0/7 |
| Group 3   | Hamster Ig, vehicle, B6 BM | Acute, median day = 11 | Acute, median day = 11 | Acute, median day = 8 | Acute, median day = 9 | 0/7 |

a Protocols were applied as outlined in Fig. 1B. All mice were preconditioned with 30 mg/kg busulfan.
b Mouse was euthanized on the indicated day with an unrelated injury.

data demonstrate a dominant role of chimerism for the inhibition of alloantibodies. In contrast to cellular immunity and transplantation tolerance, transient chimerism appears to be sufficient for the prevention of alloantibodies in this model and this persists even in the light of delayed acute skin graft rejection.

Stable hematopoietic chimerism prevents chronic heart allograft rejection in the absence of further treatment

We next tested to what extent chimerism-induced Tx tolerance could protect heart allografts as a clinically relevant solid organ. Although only ~5% of all skin allografts transplanted 3 mo after BMTx were also protected from chronic rejection, early studies by Steinmuller and Lofgreen (35) suggested that heart allografts may also constitute the only group with significant levels of IgM (23 (one outlier 125) vs 12, p = 0.029). Taken together, these data demonstrate a dominant role of chimerism for the inhibition of alloantibodies.

Table III. Lack of alloantibodies after transient chimerism with anti-LFA-1 plus everolimus treatment

| Treatment | Chimerism | BALB/c Skin Graft | IgM (mean fluorescence intensity) | IgG (mean fluorescence intensity) |
|-----------|-----------|-------------------|---------------------------------|---------------------------------|
| Group 1   | None      | Not done          | 12.1 ± 0.8 (n = 3)              | 23.8 ± 1.6 (n = 3)              |
| Group 2   | Anti-LFA-1, everolimus, BALB/c BM | Stable (Expt. 1) | Not done                         | 15.2 ± 2.7 (n = 3)              | 19.6 ± 3.5 (n = 3)  |
|           | Everolimus, BALB/c BM | None               | Not done                         | 15.0 ± 2.6 (n = 5)              | 19.5 ± 1.7 (n = 5)  |
| Group 3   | Anti-LFA-1, everolimus, BALB/c BM | Stable (Expt. 2) | Not done                         | 13.2 ± 3.4 (n = 5)              | 20.9 ± 2.7 (n = 5)  |
|           | Lost over 3 mo (Expt. 2) | Not done           | Not done                         | 13.1                           | 21.9 |
| Group 4   | Anti-LFA-1, everolimus, BALB/c BM | Stable             | Chronic from days 38, 46, 67, 74, 111, 117 | 14.0 ± 2.5 (n = 6)              | 23.0 ± 2.3 (n = 6)  |
|           | day 0, BALB/c skin day 2 | Lost over 3 mo     | Delayed acute, day 46            | 11.7                           | 23.3 |
|           |           | None              | Acute, day 18                    | 11.9                           | 80.7 |
| Group 5   | Anti-LFA-1, everolimus, B6 BM day 0, BALB/c skin day 2 | None          | Acute, median day 17             | 15.8 ± 3.8 (n = 4)              | 169 ± 84 (n = 4)    |
|           |           |                   | (n = 4)                          |                                |                                |
| Group 6   | Rat Ig, vehicle, B6 BM day 0, BALB/c skin day 2 | None         | Acute, median day 9              | 23 ± 4 (n = 3)                  | 301 ± 50 (n = 3)    |
|           |           |                   | (n = 4)                          |                                |                                | 125  578 |

a Protocols were applied as outlined in Fig. 1B. All recipients were preconditioned with 30 mg/kg busulfan. Relative IgM and IgG levels are geometric mean fluorescence intensities of BALB/c T cells stained with B6 recipient plasma harvested 8–9 mo after BMTx and FITC-conjugated anti-mouse IgM or IgG. Data are mean ± SD of the indicated number of samples.
b p = 0.029 for 4 vs 1, 3 vs 2, and 5 vs 3 by Wilcoxon-Mann-Whitney rank sum statistics.
Table IV. Stable hematopoietic chimerism prevents acute skin allograft rejection and heart allograft rejection, including protection from vascular intimal thickening (“chronic rejection”).

| Treatment for BALB/c BMTx | Chimerism Levels | BALB/c Skin Graft | BALB/c Heart Graft | Acute Heart Rejection Grade | Chronic Heart Rejection Grade |
|---------------------------|------------------|-------------------|-------------------|-----------------------------|-----------------------------|
| Group 1                   |                  |                   |                   |                             |                             |
| No busulfan, FTY720, anti-CD40L, everolimus | Low (<1% for CD3\(^+\), 2% for CD11b\(^+\)) unstable, lost by 2 mo after BMTx | Acute rejection day 55 day 16- (Fig. 5B) | n.d. | n.d. |
| Group 2                   |                  |                   |                   |                             |                             |
| No busulfan, anti-CD40L, everolimus | Low (0.5% for CD3\(^+\), 4–11% for CD11b\(^+\) cells), stable | Chronic from >day 47 >day 70\(^b\) | Intact>day 167 >day 120 (Fig. 5E) | 0 | 0 |
| Group 3                   |                  |                   |                   |                             |                             |
| 15 mg/kg busulfan, anti-CD40L, everolimus | Intermediate, stable | Chronic from >day 55 >day 120 | n.d. | n.d. |
| Group 4                   |                  |                   |                   |                             |                             |
| 30 mg/kg busulfan, FTY720, anti-CD40L, everolimus | High, stable | Chronic from day 47 >day 120 | Intact >day 167 >day 120 | 0 | 0 |
| Group 5                   |                  |                   |                   |                             |                             |
| 30 mg/kg busulfan, anti-CD40L, everolimus | High, stable | Chronic from day 26 day 43 (Fig. 5C) | Intact until >day 167 >day 120 | 0 | 0 |
| Group 6                   |                  |                   |                   |                             |                             |
| 30 mg/kg busulfan, anti-CD40L, anti-LFA-1 | High, stable | Chronic from >day 60 day 41 | Chronic from >day 51 >day 120 | 0 | 1, atrophy, fibrosis |

\(^a\) Protocols were applied as outlined in Fig. 1A.

\(^b\) Mouse was euthanized on the indicated day with an unrelated illness, graft was not harvested. Heart allografts were graded as outlined in Materials and Methods.

not pose the same problems of tissue-specific Ags as observed for skin grafts. While our experiments were ongoing, one study reported protection from acute but not from chronic heart graft rejection by established chimeras (36), whereas in another system both acute and chronic heart allograft rejection were prevented by BMTx, albeit with a protocol that included costimulation blockade beyond chimerism induction at the time of heart Tx (37). In the present study, heart allografts were transplanted 5 mo after BMTx, thus depending on established chimerism alone in the absence of peripheral immune modulation. The pretreatment regimen of some groups in early experiments included the lyophospholipid drug FTY720 which prevents allograft rejection by blocking lymphocyte egress from lymphoid organs (38). As reported for other models and combination treatments however (39, 40), FTY720 neither interfered nor synergized with combinations of anti-CD40L, anti-LFA-1, and everolimus for chimerism induction (Table IV, group 4, and data not shown). Those early BM recipients with FTY720 as part of their treatment regimen (daily from days −3 to day 0 with respect to BMTx) were included to increase overall numbers of chimeric heart allograft recipients. In addition, since levels of hematopoietic chimerism after preconditioning with 30 mg/kg busulfan tended to be very high, we also tested a smaller 15 mg/kg dose or completely omitted busulfan. Three of nine mice without busulfan conditioning developed low levels of multilineage chimerism. One recipient with low levels of chimerism that was lost by 2 mo after BMTx showed markedly delayed acute skin allograft rejection (day 55 after skin Tx), as well as delayed acute heart allograft rejection 16 days after heart Tx (Table IV, group 1). Two other BM recipients without busulfan conditioning developed very low but stable multilineage chimerism, and both were protected from acute skin graft and heart graft loss (Table IV, group 2, and Fig. 5E). One heart graft was indistinguishable from a syngeneic control graft at the 120-day end point, thus also revealing complete protection from vascular intimal thickening (“chronic rejection”). The other recipient’s heart graft was also preserved 70 days after Tx when the mouse was euthanized due to an unrelated health problem. Low to intermediate levels of chimerism (7–68% for CD11b\(^+\) and 1–18% CD3\(^+\)) were also sufficient to prevent heart allograft pathology in BM recipients conditioned with the lower dose of 15 mg/kg busulfan (Table IV, group 3). Among a total of 25 heart allografts in recipients with detectable chimerism at the time of heart Tx, 21 were completely intact and without any signs of vascular intimal thickening at the end point on day 120 after Tx. Only two heart grafts showed very mild signs of chronic rejection (grade 1) confined to one vessel and three vessels (Table IV,
groups 3 and 5). In two highly chimeric recipients, the heart allograft stopped beating around day 40 after heart Tx. Histopathological analysis revealed, however, that these failures were due to nonspecific fibrosis and myocyte atrophy, possibly as a result of prolonged ischemia time (Table IV, groups 5 and 6, and Fig. 5C).

Taken together, these data demonstrate that stable hemopoietic chimerism alone, even at very low levels, induced by previous short-term combination treatments of anti-LFA-1 plus anti-CD40L or anti-CD40L plus everolimus, protects heart allografts from acute rejection as well as vascular intimal thickening.

Discussion
In the present study, we describe novel combinations of anti-LFA-1 with anti-CD40L and everolimus that support the development of stable, mixed hemopoietic chimerism and transplantation tolerance after fully allogeneic BMTx. Combining inhibition of LFA-1 through Abs or a knockout phenotype along with CTLA-4Ig was shown to prolong heart, skin, or islet allografts, and LFA-1 blockade improved protection of skin and heart allografts in CD40L-deficient recipients or in synergy with anti-CD40L (41–44). To our knowledge, synergism between anti-LFA-1 and everolimus (or sirolimus/rapamycin) has not been reported previously to support either solid tissue or BM graft survival.

While anti-CD40L combined with anti-LFA-1 induced high incidences of stable chimerism, the combination of anti-LFA 1 plus everolimus resulted in unstable chimerism in ~50% of BM recipients. This was unlikely to be a consequence of insufficient LFA-1 blockade, because higher doses of anti-LFA-1 did not increase the incidence of chimerism stability (data not shown). Enhanced in vitro alloreactivity by responder splenocytes from recipients with unstable chimerism was consistent with host rejection of donor hemopoietic cells rather than passive loss of engraftment. In contrast to increased cellular alloreactivity, however, slow loss of chimerism did not induce alloantibody production, indicating differential regulation of T and B cell alloimmunity by BMTx in this system. BM recipients without any detectable chimerism did not develop alloantibodies unless challenged with a skin allograft, suggesting that in this situation B cells were neither activated nor tolerized by allogeneic BM alone. Recipients with stable mixed chimerism contained no alloantibodies with or without skin allografts. Formally, this might have been accounted for by indirect inhibitory effects on B cells through T cell tolerance. Surprisingly, unstable chimerism after anti LFA-1 plus everolimus treatment did not trigger alloantibody production even after acute skin allograft rejection. Together, these observations suggest that in this system 1) loss of chimerism resulted from cellular alloreactivity alone; 2) lack of alloantibodies after transient and (probably also stable) chimerism reflected direct B cell tolerance; 3) humoral effector mechanisms were not required for acute skin allograft rejection; 4) B cell tolerance through transient chimerism was not broken by acute skin allograft rejection; and 5) T and B cell compartments have different requirements for tolerance induction after BMTx, and direct B cell tolerance is possible in the absence of T cell tolerance. T cell-independent intrinsic B cell tolerance was previously described for a mouse model of semiallogeneic BMTx. In this study, B cell tolerance required the presence of semiallogeneic hemopoietic cells, as it was observed in mixed but not in full bone marrow chimeras (45). Although our data suggest persisting B cell tolerance after loss of chimerism, this does not
argue against the requirement for the continuous supply of a hematopoietic source of alloantigen, which may be present below the detection limit of flow cytometry and/or in distinct locations such as the bone marrow. In contrast to our mouse model, alloantibodies may occur after loss of chimerism and contribute to solid organ allograft rejection in nonhuman primate models that are closer to the clinical setting (34). It will be important to investigate further the role of humoral alloreactivity in the context of BMTx, the requirements for B cell tolerance, and to identify regimens that induce and maintain this process. Interestingly, the combination treatment of anti-LFA-1 plus everolimus alone without allogeneic BM reduced alloantibody levels after acute skin allograft rejection. It was not clear, however, whether this was secondary to transiently reduced cellular immunity (as indicated by a delay in skin graft rejection) or the consequence of a direct effect on B cells.

Notwithstanding the suboptimal average rate of ~50% incidence of stable chimerism after treatment with anti-LFA-1 plus everolimus, the data show that, in principle, this combination can provide sufficient immune modulation to protect fully allosmached hematopoietic cells, albeit with a lower probability. Although costimulation through LFA-1 was shown to contribute to IL-2 production, cell cycle progression, and effector functions of both CD8 and CD4 T cells (46–49), CD8 T cells appeared to be more sensitive to LFA-1-mediated costimulation (50, 51). A recent study using allogeneic hematocyte transfer in CD4- and CD8-deficient mice demonstrated the relevance of both CD40L and LFA-1 costimulation pathways for both alloreactive CD4 and CD8 T cells, albeit with a stronger influence of LFA-1 than CD40L on costimulation for CD4-independent CD8-dependent alloreactivity (52). In the context of hematopoietic chimerism, selective CD4 and CD8 depletion experiments revealed that anti-CD40L could overcome CD4 but not CD8 T cell-dependent resistance to allogeneic BM engraftment (53). Everolimus, like rapamycin/sirolimus, interferes with advanced stages of T cell activation by inhibiting growth factor responsiveness (54). Although this might be expected to inhibit both CD4 and CD8 T cells, rapamycin appeared to affect CD8 T cell alloreactivity and proliferation more strongly (55, 56). Taken together, it is conceivable that the combinations of anti-CD40L plus anti-LFA-1 and anti-CD40L plus everolimus provide sufficient blockade of both CD4- and CD8-dependent alloreactivity, whereas the combination of anti-LFA-1 plus everolimus may be suboptimal for blocking CD4 T cells. Ongoing experiments address the effect of different combinations on CD4 and CD8 T cell alloreactivity.

Rapamycin/sirolimus was recently shown to induce hematopoietic chimerism in combination with anti-CD40L, albeit in the context of more permissive protocols that also supported chimerism with rapamycin or anti-CD40L alone (57) or with CD40L blockade alone (58). By contrast, under the relatively stringent conditions used in our studies (no irradiation, no donor-specific transfusion, single busulfan treatment, and single BM cell transfer), the combination of anti-CD40L and everolimus was strictly synergistic, as neither reagent alone supported chimerism upon BMTx. This contrasts with reports on BMTx-induced chimerism under CD40L blockade alone, presumably owing to stronger cyto reduction or repeated BM infusion with more frequent administration of anti-CD40L (12, 56, 57). Several mechanisms of action were proposed for anti-CD40L, as a single agent or in combination with CTLA4lg or rapamycin, including the induction of T regulator cells (58, 59), Fe-and complement-dependent depletion (60, 61), and apoptosis through costimulation blockade (10, 62, 63). In our experiments, short-term treatment with anti-CD40L combined with either anti-LFA-1 or everolimus did not prevent acute skin allograft rejection in the absence of allomatched BMTx. Nor was acute rejection of secondary skin grafts delayed, thus demonstrating a lack of peripheral allogeneic BM-independent mechanisms of tolerance. Despite the relatively short-lived protection in the absence of allospecific BMTx, BALB/c skin grafts transplanted 2 days after BALB/c BMTx were well protected with either anti-CD40L-containing combination treatment. Since central deletion of allospecific thymocytes during this early period after BMTx was unlikely to be sufficient for allograft tolerance, it is possible that allogeneic BM-dependent peripheral deletion of donor-reactive T cells, as reported previously (10), could have contributed to skin allograft protection. Compared with pretransplant Vβ11+ data and those measured for recipients of syngeneic BM, we observed a moderate reduction of Vβ11+ TCR T cells from 5–6% to 4–5% in anti-CD40L plus anti-LFA-1 and anti-CD40L plus everolimus-treated recipients 2 wk after allogeneic BMTx in these experiments (data not shown). When skin Tx was performed 3 mo after BMTx and in the absence of peripheral immune modulation however, the clear correlation between substantial host-type Vβ11+ T cell depletion and chimerism stability and between chimerism stability and transplantation tolerance strongly suggested that thymus-independent mechanisms of tolerance, if they existed under these conditions, were not sufficient to prevent acute allograft rejection.

A recent study demonstrated a correlation among T cell chimerism, host Vβ11+ T cell depletion, and skin graft tolerance (65). Our data also strongly implied yet could not systematically show a critical role for T cell chimerism, because we rarely observed selective loss of T cell chimerism while developing or preserving chimerism among other hematopoietic lineages.

In all our experiments, nonmyeloablative cytoreduction before BMTx was achieved with busulfan, initially at a dose of 30 mg/kg previously shown to support high levels of chimerism (13). Even the highest busulfan dose did not support chimerism in the absence of potent costimulation blockade however, indicating that busulfan was ineffective as an immunosuppressive agent in this system as it was in previous murine studies (13). This may differ for haploidential settings, as shown in a dog model where an incidence of 50% chimerism with busulfan treatment alone was reported for MHC-compatible BM donors (66). However, as also reported in this study, attempts to induce chimerism with MHC-mismatched host-donor combination failed with busulfan alone, suggesting a minor role of busulfan as an immunosuppressive agent compared with its more dominant cyto reduce function.

Because of series side effects in humans, the clinical use of busulfan for BM (or stem cell) Tx and cancer treatment is usually limited to 16 mg/kg or less, given through multiple applications over several days (67, 68). Therefore, ideally, novel immune modulatory regimens should be effective when combined with milder cytoreduction. In our murine system, effective immune modulation after reduced busulfan dosing (15 mg/kg) induced high incidences of lower overall levels of hematopoietic chimerism, yet without compromising transplantation tolerance. Moreover, it was possible to achieve low incidences of stable multilineage chimerism with potent immune modulation when busulfan was omitted completely. Presumably, strong inhibition of host-anti-donor BM alloreactivity permits some donor stem cells to settle within host BM niches during physiological stem cell migration in the absence of drug- or irradiation-induced homeostasis perturbation (69). A competitive advantage of allogeneic BM stem cells with ensuing mixed chimerism in the absence of cyto reduce conditioning was also achieved with costimulation blockade and multiple standard doses (2 × 10^6) or a single high dose (2 × 10^6) of allogeneic BM cells (11, 12).
Although stable hemopoietic chimism, even at very low levels, prevented acute skin graft rejection, most recipients nevertheless displayed signs of chronic skin graft deterioration of variable and usually late onset and in the light of fully preserved hemopoietic chimism, as described previously for immunity against BM-independent skin-specific Ags (29–32, 70). Heart allograft integrity, however, was never compromised in chimeric recipients with chronic skin graft rejection, suggesting lack of cross-reactivity between skin and heart Ags. Similar conclusions were drawn from earlier studies that compared heart and skin graft survival in radiation chimeras, although histological screening for heart arteriopathy was not reported (35). Although a short 2-day interval between BMTx and skin Tx substantially delayed or prevented chronic rejection, it did not achieve complete protection. This contrasts with studies by Adams et al. (13), who demonstrated indefinite skin allograft survival for the same BALB/c–B6 donor–recipient combination when BMTx and skin Tx were performed on the same day under costimulation blockade with anti-CD40L and CTLA-4Ig administered until day 28. Conceivably, costimulation blockade over a longer period and/or with still closer timing of BMTx and skin Tx improved the incidence of complete skin allograft protection. Alternatively or in addition, CTLA-4Ig might have increased the probability of indefinite skin graft survival, possibly by superior costimulation blockade and/or by promoting apoptosis of skin Ag-specific T cells through B7-induced modulation of APC tryptophan metabolism (71).

In our experiments, stable hemopoietic chimism, even at very low levels, was sufficient to prevent both acute and chronic heart graft rejection, as previously reported for a rat model of mixed chimism (72). Costimulation blockade and allogeneic BM-mediated protection from chronic heart graft rejection in mice was recently reported by Shiragusi et al. (37). In their study, BMTx and heart Tx were performed on the same day, with both grafts being under the effect of costimulation blockade with anti-CD40L and CTLA-4Ig until 3 mo after Tx. It was possible, therefore, that in this case, heart allografts benefited from concurrent BMTx with extended costimulation blockade. In contrast, in another mouse model, Russell et al. (36) observed chronic rejection of heart grafts transplanted to established chimeras rather than at the time of BMTx. This suggests that receptor blockade might be less effective for heart rejection than graft rejection in the model of Russell et al. (36), where the use of CTLA-4Ig and anti-CD40L during the peri-transplant period in one but not the other regimen. Neither of these explanations readily account for the lack of chronic rejection in our experiments however, since stable, mixed hemopoietic chimism through BMTx under short-term combination treatments of anti-CD40L and anti-LFA-1 or everolimus was sufficient to fully preserve heart allografts transplanted ~5 mo after BMTx. Possibly, variable success of complete allograft protection in hemopoietic chimeras may be related to initial conditioning regimens and/or immune modulation as well as different "shades" of tissue-specific Ag immunogenicity between different donor-recipient strain combinations. For a clinically relevant approach, it will now be necessary to further understand the requirements for immune modulation that support chimism stability and to identify the critical parameters that determine complete allograft integrity in chimergic recipients.

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