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Anti-LFA-1 or rapamycin overcome costimulation blockade-resistant rejection in sensitized bone marrow recipients

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Summary

While costimulation blockade-based mixed chimerism protocols work well for inducing tolerance in rodents, translation to preclinical large animal/nonhuman primate models has been less successful. One recognized cause for these difficulties is the high frequency of alloreactive memory T cells (Tmem) found in the (pre)clinical setting as opposed to laboratory mice. In the present study, we therefore developed a murine bone marrow transplantation (BMT) model employing recipients harboring polyclonal donor-reactive Tmem without concomitant humoral sensitization. This model was then used to identify strategies to overcome this additional immune barrier. We found that B6 recipients that were enriched with 3×10^7 T cells isolated from B6 mice that had been previously grafted with Balb/c skin, rejected Balb/c BM despite costimulation blockade with anti-CD40L and CTLA4Ig (while recipients not enriched developed chimerism). Adjunctive short-term treatment of sensitized BMT recipients with rapamycin or anti-LFA-1 mAb was demonstrated to be effective in controlling Tmem in this model, leading to long-term mixed chimerism and donor-specific tolerance. Thus, rapamycin and anti-LFA-1 mAb are effective in overcoming the potent barrier that donor-reactive Tmem pose to the induction of mixed chimerism and tolerance despite costimulation blockade.

Introduction

Numerous treatment protocols inducing mixed hematopoietic chimerism lead to robust transplantation tolerance in rodents [1]. Recently, the mixed chimerism strategy also led to operational tolerance in most participants of clinical proof-of-principle trials [2,3]. Translation of less toxic and thus clinically more acceptable, experimental chimerism protocols, however, has been associated with substantial setbacks [4]. While permanent mixed chimerism can be induced in rodents with minimal conditioning [5–9], long-term chimerism in large animals is much more difficult to achieve [10]. In nonhuman primates, in particular, macrochimerism is usually detectable only transiently despite recipient conditioning regimens that are substantially more intense (i.e., cytotoxic and myelotoxic) than those used in rodents [11]. Notably, costimulation blockade, which is sufficient for inducing mixed chimerism in nonmyeloablated mice [12–14], fails to establish chimerism in MHC-mismatched nonhuman primates [15,16]. Moreover, while mixed chimerism induces B cell tolerance in rodents [17], evidence for B cell immunity – albeit of unknown clinical consequence – was observed in the clinical setting [2,18].

The different frequencies of memory T cells (Tmem) in patients and nonhuman primates on one side and (usually young) laboratory mice kept under protected conditions on the other side has emerged as a critical factor accounting for the difficulty in translating tolerance protocols from

bench to bedside [19,20]. Tmem respond more rapidly to antigen recognition, are less dependent on 'conventional' costimulation pathways (i.e., CD28 and CD40) and are more resistant to regulation than naïve T cells [21]. Even in the absence of previous exposure to alloantigen, alloreactive Tmem are generated through heterologous immunity and homeostatic proliferation [22,23]. Humoral sensitization has been investigated in murine mixed chimerism models and has been recognized as sizable barrier [24,25]. While humoral sensitization can be generally avoided in renal transplantation through pretransplant cross-match assays detecting donor-specific antibodies (DSA), no assays for evaluating sensitization at the T cell level are yet available for routine use in the clinical setting [26]. Such T cell sensitization causes costimulation blockade-based tolerance protocols developed in naïve mice to fail when applied to recipients enriched with T cells sensitized to the donor [27]. Likewise, concomitant or previous exposure to infections leads to the failure of otherwise successful costimulation blockade-based murine mixed chimerism protocols owing to heterologous immunity [22,28]. In allosensitized recipients, Tmem were shown to persist even after lethal irradiation (10 Gy) mediating rejection of BM [25]. It remains to be determined, however, if and how an isolated Tmem barrier without concomitant humoral sensitization can be overcome in recipients of a costimulation blockade-based mixed chimerism regimen.

To closer model the clinical setting, we therefore developed a nonmyeloablative murine protocol of mixed chimerism in which recipients contain functionally relevant numbers of polyclonal alloreactive Tmem. Moreover, we demonstrate that anti-LFA-1 and rapamycin are effective in controlling Tmem reactivity in this new BMT model.

Materials and methods

Animals

Female C57Bl/6 (B6, H-2^b, CD45.2), Balb/c (H-2^d), and C3H/N (H-2^k) mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Congeneic CD45.1 recipients (B6.SJL-Ptprc Pepc/BoyJ) were purchased from Charles River Italy (Calco, Italy). All mice were housed under specific pathogen-free conditions and were used between 6 and 10 weeks of age. All experiments were approved by the local review board of the Medical University of Vienna and were performed in accordance to national and international guidelines of laboratory animal care.

Skin grafting

Donor Balb/c or third party C3H/N full thickness tail skin was grafted onto naïve B6 mice or B6 BMT recipients (at least 6 weeks post-BMT) for either means of Tmem generation or tolerance assessment, respectively. Grafts were visually inspected daily and considered to be rejected when less than 10% remained viable.

Detection of donor-specific antibodies (DSA)

Serum was collected from skin recipients and heat deactivated before incubation with thymocytes from B6, Balb/c, and C3H/N mice. After a 30 min incubation period, cells were washed and labeled with FITC-anti-mouse $IgG_{1/2}$ (BD Pharmingen, San Diego, CA, USA) to detect cell-bound anti-donor antibodies (DSA) via flow cytometric analysis.

Generation and isolation of Tmem cells

Three weeks after skin grafting, mice were tested by flow cytometry for DSA. T cells were isolated from spleen and lymph nodes of DSA-positive mice 3 weeks post skin grafting through MACS separation (Pan T cell Exclusion Kit; Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of separated cells was >96%.

Mixed lymphocyte reaction (MLR)

Mixed lymphocyte reactions were performed as described previously [7]. Briefly, 4×10^5 responder splenocytes were plated in triplicates and incubated with 4×10^5 irradiated (30 Gy) stimulator cells of either Balb/c (donor), C3H (third party), B6 (host) mice, or with medium only. After 72 h, cells were pulsed with ^{3}H-thymidine (Amersham Biosciences, Sunnyvale, CA, USA) and incubated for approximately 18 h. Incorporated radioactivity was measured using scintillation in a β-counter. Stimulation indices (SI) were calculated in relation to medium controls.

BMT protocol

Two or 3 Gy TBI was administered to B6 recipients 1 day before receiving 20×10^6 unseparated Balb/c bone marrow cells (BMC) and costimulation blockade (1 mg of MR1 hamster anti-mouse-CD40L (CD154) mAb on day 0 and 0.5 mg of human CTLA4Ig (abatacept) on day 2) [7]. Where indicated groups received in addition either rapamycin (0.1 mg d-1, d0, and d2) (Alexis Biochemicals, San Diego, CA, USA) or blocking anti-LFA-1 mAb (M17/4) (0.5 mg, d-1 and d2). Anti-CD40L and anti-LFA-1 mAbs were purchased from BioXCell (West Lebanon, NH, USA) and hCTLA4Ig was generously provided by Bristol-Myers, Squibb Pharmaceuticals (Princeton, NJ, USA).

Flow cytometric analysis of chimerism, deletion, and intracellular IFNγ expression

Two-color flow cytometric analysis was used to distinguish donor and host cells of particular lineages by staining with fluorescin isothiocyanite-conjugated antibodies against CD4, CD8, B220, MAC1, and biotinylated antibody against H-2D^d (34-2-12, developed with phycoerythrin streptavidin) and isotype controls. To analyze the expression of Vβ subunits among splenocytes, staining was performed with fluorescin isothiocyanite (Fitc) antibodies against Vβ8.1/2, Vβ11, and Vβ5.1/2 (or isotype control) and phycoerythrin-conjugated (PE) antibodies against CD4 and CD8 (antibodies from Becton Dickinson, San Diego, CA, USA and Biolegend, San Diego, CA, USA). To analyze Vβ subunits in the thymus, Vβ8.1/2, Vβ11, and Vβ5.1/2 (or isotype control) expression was measured on gated single positive thymocytes (CD4$^+$ [PE] CD8$^-$ [Cy5] and CD4$^-$ [PE] CD8$^+$ [Cy5]). Propidium iodide staining was used to exclude dead cells. The net percentage of donor chimerism was calculated by subtracting control staining from quadrants containing donor and host cells expressing a particular lineage marker, and by dividing the net percentage of donor cells by total net percentage of donor plus host cells of that lineage. Mice were considered chimeric if they showed at least 2% donor cells within the myeloid lineage and within at least one lymphoid lineage [7,29,30]. Intracellular staining for IFN-γ was performed as per the manufacturer's instructions (Cytofix/Cytoperm kit; Biolegend). Cells from spleen or lymph nodes were resuspended in cell culture media containing brefeldin (GolgiPlug; BD Pharmingen) and plated in 96-well plates at a 1:1 ratio with irradiated donor splenocytes (1×10^6 cells per well) and restimulated for 3–4 h. Cells were washed and analyzed by staining with fluorochrome-conjugated antibodies (Biolegend) to CD4 and CD8, followed by intracellular staining with Alexa Fluor® 647 anti-mouse IFN-γ (Biolegend). Flow cytometric analysis was done on a Coulter Cytomics FC500. CXP software (Coulter, Brea, CA, USA) was used for acquisition and analysis.

Histological analysis

Sections of 4 μm were cut from paraffin-embedded tissue fixed in 4.5% formalin (with a buffered pH of 7.5), stained with hematoxylin–eosin and Giemsa according to standard protocols, and analyzed by an experienced pathologist.

ELISPOT

IFN-γ secretion was induced in response to 18–20 h of *ex vivo* stimulation with allogeneic stimulators. In brief, splenocytes or lymph nodes were prepared from experimental and naive animals, responders were resuspended in cell culture media and plated in triplicates in 96-well PVDF Membrane ELISPOT plates (Millipore, Billerica, MA, USA) at a 2:1 ratio (8×10^5 responder cells per well) with 4×10^5 stimulators (host, donor, or third party) or medium alone. The mouse IFN-γ ELISPOT kit (eBioscience, Frankfurt, Germany) was used according to the manufacturer's instructions, freshly prepared AEC substrate (Sigma, St. Louis, MO, USA) was added for 10 min at room temperature for spot development. Analysis was performed on a Bioreader 5000 (BIOSYS, Pasadena, CA, USA) with Bioreader 10.8 software. Spot development was calculated in relation to medium controls.

Statistical analysis

For comparing the rates of chimerism between groups Fisher's exact test was used. A two-tailed Student's *t*-test was used for comparing Vβ deletion and levels of chimerism between groups. A value of $P < 0.05$ was considered to be statistically significant. Survival was calculated according to the Kaplan–Meier product limit method and compared between groups using the log-rank test. Graph Pad Prism software was used for creating Kaplan–Meier survival curves.

Results

Enriching BMT recipients with donor-reactive Tmem abrogates chimerism and tolerance despite costimulation blockade

First, a model mimicking the clinical situation of T cell sensitization without accompanying humoral sensitization was established (Fig. 1a). Naïve B6 mice were grafted with Balb/c skin and upon confirmation of the development of donor-specific antibodies 3 weeks postgrafting (indicative for T cell sensitization) (Fig. 1b), T cells were isolated for transfer into naïve B6 mice (which went on to serve as BMT recipients). These cells showed significantly increased reactivity to Balb/c ($P < 0.01$), but not third party C3H ($P = 0.085$) stimulators in *in vitro* MLR assays (Fig. 1c) in comparison to naïve BL6 cells. (*Note*: for reasons of simplicity the transferred cell population is referred to as 'Tmem' hereafter.) Similarly, freshly isolated Tmem cells reacted with substantially higher levels of IFN-γ secretion in response to donor (but not third party antigen) in ELISPOT assays as compared to naïve T cells (Fig. 1e). In addition, intracellular IFN-γ production was increased among Tmem upon polyclonal and donor-specific stimulation (Fig. 1f). Phenotypic analysis of Tmem cells by flow cytometry revealed an increased percentage of CD4 and CD8 effector and central memory cells in comparison to naïve T cells (Fig. 1d).

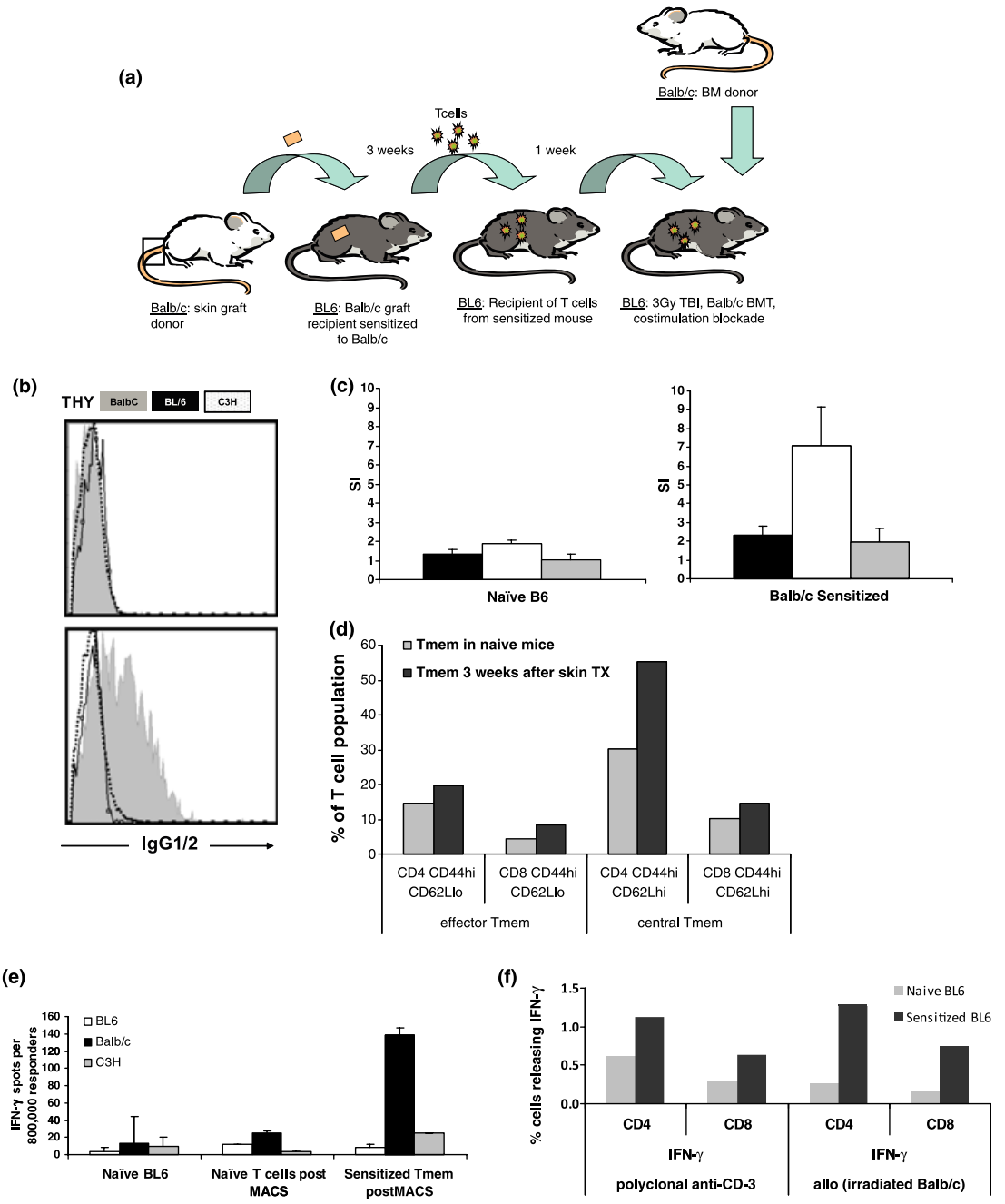

Figure 1 Generation of a BMT model using Tmem-enriched recipients. (a) Naïve B6 mice were grafted with Balb/c tail skin to sensitize them toward Balb/c. Three weeks later, T cells were isolated through magnetic bead separation and transferred to another set of naïve B6 mice that served as recipients of Balb/c BM 1 week later. (b) To ensure that T cells are only transferred after successful sensitization, serum from each skin grafted mouse was tested by flow cytometry for the presence of high levels of donor-specific antibodies (IgG1/2) against Balb/c (gray filled curve), B6 (black curve), and C3H (dotted curve) thymocytes (shown in comparison to serum from a naïve B6 control – upper panel). Results from one representative mouse are shown. (c) In MLR assays, T cells isolated from sensitized mice showed increased proliferation in response to Balb/c stimulators, but not to C3H third party stimulators (compared with naïve B6). Mean stimulation indices (SI) of recipient responder cells against B6 (n = 3) (black), Balb/c (n = 3) (white), and C3H/N (n = 2) (gray) stimulator are shown. Error bars indicate standard deviation. (d) Phenotypic analysis of pooled isolated Tmem cells (n = 3) showed an increase in Tmem specific markers in both CD4/8 effector and central memory cells. (e) IFN-γ specific ELISPOT analysis shows enhanced donor-specific memory responses with MACS separated pan T cells isolated from sensitized mice in comparison to pan T cells from naïve mice and unsorted splenocytes from naïve mice (n = 2 per group). Responses against B6 (white), Balb/c (black), and C3H (gray) stimulators are shown. (f) Frequency of intracellular IFNγ responses in $CD8^+$ and $CD4^+$ splenocytes from pooled sensitized (dark gray, n = 2) and pooled naïve mice (light gray, n = 2) in response to donor antigen are shown.

Collectively, these data provide evidence that the 'Tmem' population generated for use in this model show phenotypic and functional properties characteristic of memory/effector T cells.

Increasing numbers of sensitized T cells were administered to B6 mice which 1 week later underwent BMT (15–20 $\times$ 10^6 Balb/c BMC, 2 or 3 Gy TBI, and costimulation blockade). Mice receiving 1 $\times$ 10^5 Tmem cells (4/5 chimeric, not shown), 1 $\times$ 10^6 (3/4 chimeric, not shown), or 1 $\times$ 10^7 (3/5 and 4/5 chimeric) after 2 Gy TBI, and mice receiving 2 $\times$ 10^7 Tmem cells after 3 Gy TBI showed no significant abrogation of chimerism in comparison to the respective BM control groups (3/5 and 5/8 chimeric) (Fig. 2a). Transfer of 2 $\times$ 10^7 Tmem cells after 2 Gy (0/5 chimeric) and 3 $\times$ 10^7 Tmem after 3 Gy abrogated chimerism (0/4 chimeric) (Fig. 2a). Results from multiple repeat experiments confirmed that the transfer of 3 $\times$ 10^7 Tmem after 3 Gy TBI reproducibly abrogated multi-lineage chimerism in the majority of recipients (6/32 chimeras in recipients enriched with Tmem compared with 29/37 chimeras in control BMT recipients without T cell transfer, pooled data from seven experiments, $P < 0.01$) (Fig. 2b and d). Moreover, BMT recipients enriched with 3 $\times$ 10^7 Tmem rejected donor skin whereas most recipients without cell transfer accepted donor skin for a long term (while rejecting third party grafts, not shown) (Fig. 2c). A total of 3 $\times$ 10^7 transplanted Tmem consist of 52.6% CD4 (15 $\pm$ 2 $\times$ 10^6 cells) and 45.7% CD8 (13 $\pm$ 1 $\times$ 10^6 cells), close to a 1:1 ratio. When either 1.5 $\times$ 10^7 $CD4^+$ or 1.5 $\times$ 10^7 $CD8^+$ cells isolated from sensitized mice were transferred into BMT recipients (which is comparable to the amount of CD4 and CD8 cells contained in bead-separated 3 $\times$ 10^7 T cells as assessed by flow cytometry), chimerism was not abrogated (4/6 and 3/4 chimeric; 3 Gy TBI; data not shown), indicating that it is neither solely the CD4 subset nor solely

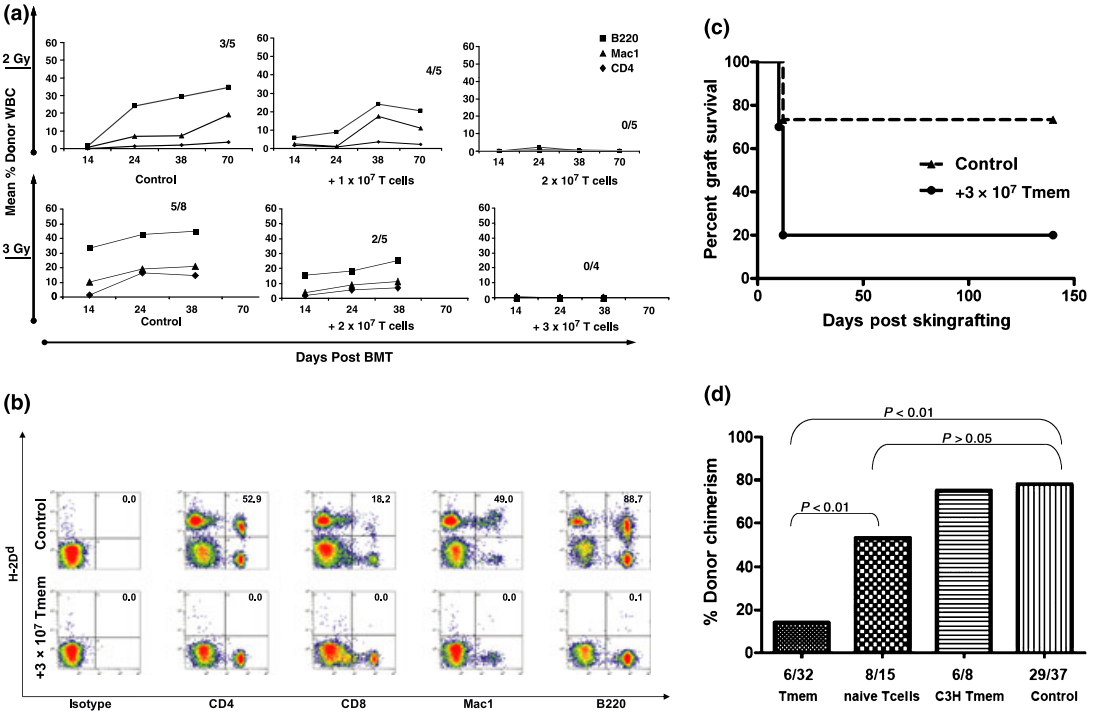

Figure 2 Enriching BMT recipients with donor-reactive Tmem abrogates chimerism and tolerance despite costimulation blockade. (a) Increasing numbers of sensitized T cells were administered to B6 mice which 1 week later underwent BMT (15 $\times$ 10^6 Balb/c BMC with 2 Gy or 20 $\times$ 10^6 BMC with 3 Gy TBI and costimulation blockade). Chimerism was abrogated when 2 $\times$ 10^7 Tmem (2 Gy) and 3 $\times$ 10^7 Tmem cells (3 Gy) were transferred. Mean percentage chimerism among various leukocyte lineages in blood is shown over time as determined by flow cytometry. Levels of mean donor B220 (■), Mac1 (▲), and CD4 (♦) chimerism are shown over time. Numbers of chimeric mice are denoted at the end of follow-up. (b) Two-color flow cytometric analysis of multi-lineage chimerism is shown for a representative BMT recipient without cell transfer (top) and a BMT recipient enriched with 3 $\times$ 10^7 Tmem (bottom) (8 weeks post-BMT). Numbers indicate net percentage of donor chimerism in each lineage. (c) Tmem and control mice were grafted with Balb/c donor skin. While most control BMT recipients without cell transfer (broken line, ▲, $n = 15$) accepted donor grafts long-term, Tmem-enriched BMT recipients rapidly rejected donor skin (solid line, ●, $n = 10$ $P = 0.0074$). (d) T cells isolated from mice sensitized to C3H, from mice sensitized to Balb/c ('Tmem'), T cells from naïve mice or no T cells were transferred to BMT recipients. The percentage of mice that became chimeric with each of these protocols is shown. Data are shown from a total of nine separate experiments with each experimental group being performed at least two times.

the CD8 subset contained in 3×10^7 Tmem that causes rejection. To investigate whether transferred Tmem need to be donor-specific to abrogate chimerism, T cells from B6 mice grafted with C3H skin were used for adoptive transfer. Transfer of Tmem from C3H sensitized mice did not significantly reduce the rate of chimerism (6/8 vs. 29/37 without cell transfer, $P = 0.34$), neither did the transfer of T cells isolated from naïve B6 mice [8/15 chimeras vs. 29/37 (BMT control), $P = 0.055$; vs. 6/32 (Tmem), $P = 0.037$] (Fig. 2d). Consequently, the transfer of

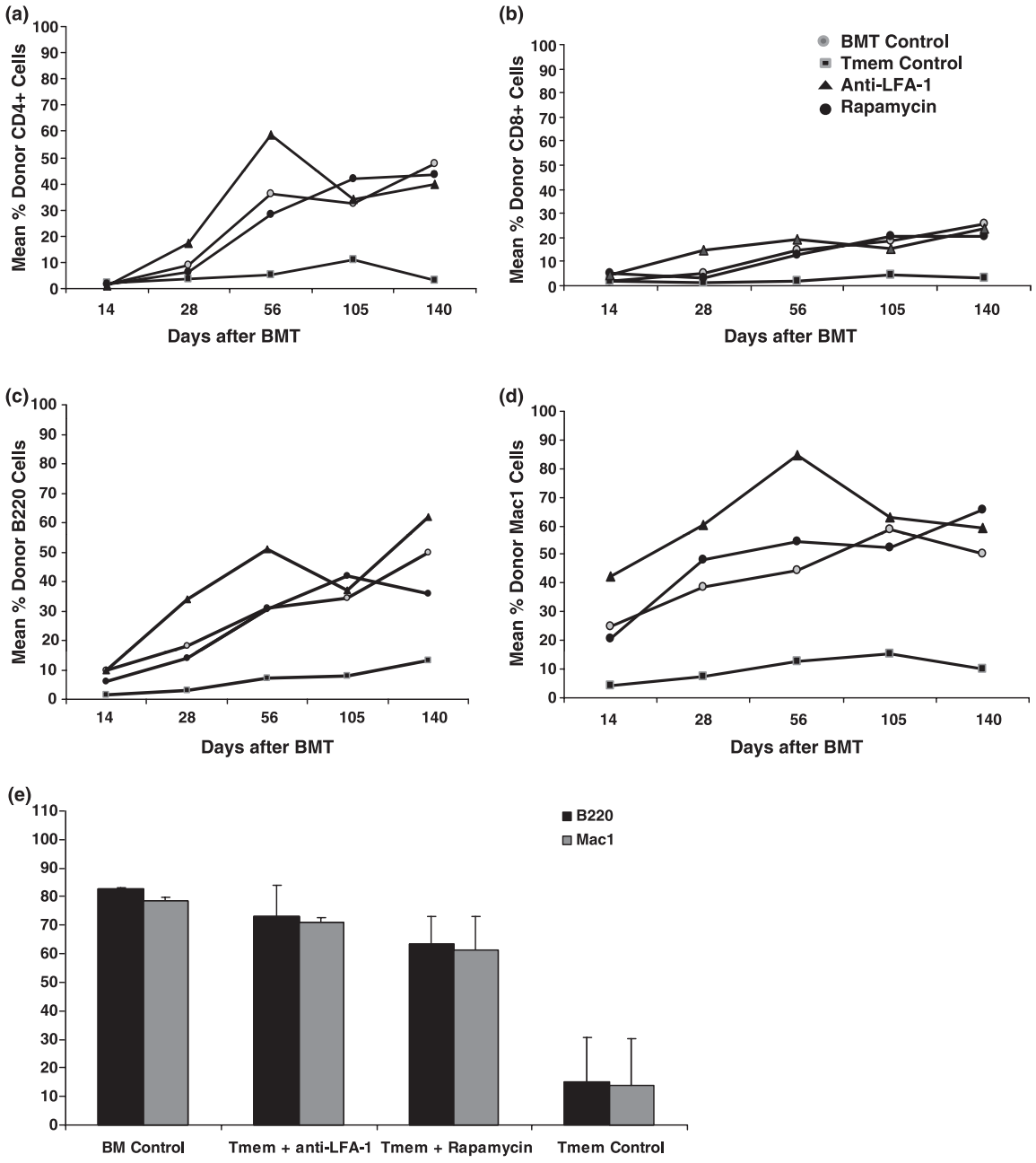

Figure 3 Stable long-term multi-lineage hematopoietic chimerism in presensitized BMT recipients after administration of additional anti-LFA-1 or rapamycin. Mean percent chimerism among CD4 cells (a), CD8 cells (b), B cells (c) and myeloid cells (d) is shown over time in peripheral blood for Tmem-enriched BMT recipients treated in addition with anti-LFA-1 (Δ, $n = 14$), rapamycin (○, $n = 14$) or no additional treatment (Tmem control, ■, $n = 31$) (compared with BMT recipients without cell transfer (BMT control, ●, $n = 37$) All groups received costimulation blockade. (e) Mean levels of B cell (black bars) and myeloid (gray bars) chimerism within bone marrow of anti-LFA-1 ($n = 10$) and rapamycin-treated Tmem-enriched recipients ($n = 6$) were not significantly different in comparison to those of BMT recipients without cell transfer ($n = 9$; 20 weeks post-BMT) (Tmem control $n = 6$).

3×10^7 Tmem sensitized to Balb/c together with 3 Gy TBI was chosen for subsequent investigations.

Thus, we have developed a model in which BMT recipients enriched in donor-reactive Tmem cells reject donor BM despite costimulation blockade, preventing the development of chimerism and tolerance.

Stable long-term multi-lineage hematopoietic chimerism in presensitized BMT recipients after administration of additional anti-LFA-1 or rapamycin

Next, we aimed to identify drugs that would control Tmem cells in BMT recipients overcoming T cell sensitization. When LFA-1 – which is upregulated on Tmem and whose blockade acts synergistically with costimulation blockade [36,37] – was blocked with an anti-LFA-1 mAb at the time of BMT (0.5 mg, d-1 and d2), long-term (>3 months), multi-lineage chimerism was induced in the majority of Tmem-enriched BMT recipients (10/14, $P < 0.001$) (Fig. 3a –d). Likewise, when rapamycin – which has multiple mechanisms of action with regard to naïve, regulatory, and memory T cells [38] – was used as adjunctive treatment (0.1 mg d-1, d0, and d2), chimerism was successfully established in Tmem-enriched BMT recipients (12/14, $P < 0.001$) (Fig. 3a –d). Chimerism rates achieved in Tmem-enriched recipients treated with anti-LFA-1 or rapamycin were comparable with those observed in BMT recipients without cell transfer (29/37, $P = 0.2$ and 0.3), indicating that the additional engraftment-inhibiting barrier of Tmem was successfully overcome. Successful chimerism induction was also evident in BM in rapamycin- and anti-LFA-treated mice at the end of follow-up (Fig. 3e).

Tolerance in presensitized BMT recipients after administration of additional anti-LFA-1 or rapamycin

To assess whether anti-LFA-1 or rapamycin-treated chimeras developed donor-specific tolerance, donor Balb/c and C3H tail skin was grafted ca 6 weeks post-BMT. Balb/c skin survived long-term (Fig. 4a) while third party skin was rapidly rejected in all groups (MST = 12 days, data not shown). Histological analysis at the end of follow-up revealed that donor grafts of Tmem-enriched recipients were comparable to BMT recipients without cell transfer in that they showed negligible eosinophil and neutrophil infiltration and only moderate intraepithelial infiltrates (data not shown).

In vitro MLR assays were performed at the end of the follow-up (8–20 weeks post-BMT). As shown in Fig. 4b, both rapamycin and anti-LFA-1-treated BMT recipients demonstrated donor-specific hyporesponsiveness. Taken together, these data reveal that sensitized BMT recipients additionally treated with anti-LFA or rapamycin develop donor-specific tolerance.

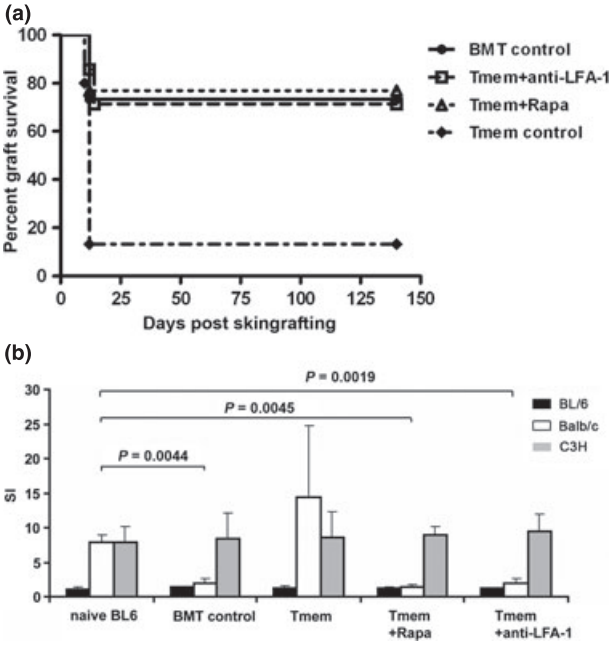

Figure 4 Tolerance in presensitized BMT recipients after administration of additional anti-LFA-1 or rapamycin. (a) Anti-LFA-1 (□, dashed line, $n = 14$) and rapamycin- (Δ, dotted line, $n = 13$) treated groups showed donor skin graft survival comparable to BMT recipients without cell transfer (●, solid line, $n = 15$) whereas Tmem-enriched BMT recipients without additional treatment rapidly rejected donor grafts (♦, dash-dot line, $n = 15$). (b) Tmem-enriched BMT recipients treated with rapamycin or anti-LFA-1 showed donor-specific hyporesponsiveness in MLR assays performed 8 weeks post-BMT ($P = 0.0045$ for Rapa, $P = 0.0019$ for anti-LFA-1; SI anti-donor compared with naïve B6 mice; $P = 0.1593$ for Rapa, $P = 0.1709$ for anti-LFA-1 compared with Tmem control; $n = 3$ for each group). Mean SI indices of recipient responder cells against B6 (black), Balb/c (white), and C3H/N (gray) stimulator are shown. Error bars indicate standard deviation.

Transferred memory T cells do not proliferate in BMT recipients

To follow adoptively transferred cell populations post-BMT, CD45.2 B6 Tmem or naïve T cells were transferred into CD45.1 B6 recipients. Nine days post-BMT (i.e., 16 days post T cell transfer) no evidence for proliferation of transferred T cells in response to donor BMT was found in either treated or untreated groups as levels of CD45.2 cells in blood were not increased over baseline (2 days before BMT, i.e., 5 days post-transfer) (Fig. 5a). Overall, levels of CD45.2 T cells were not statistically different between groups. However, a trend toward higher numbers of persisting Tmem (in particular $CD4^+$) was observed with rapamycin and anti-LFA-1 (compared with untreated Tmem recipients) (Fig. 5b). Analysis of spleen and lymph nodes revealed similar results with no statistical significance between Tmem-enriched groups with or without additional anti-LFA-1 or rapamycin. Thus, we think that it is unlikely

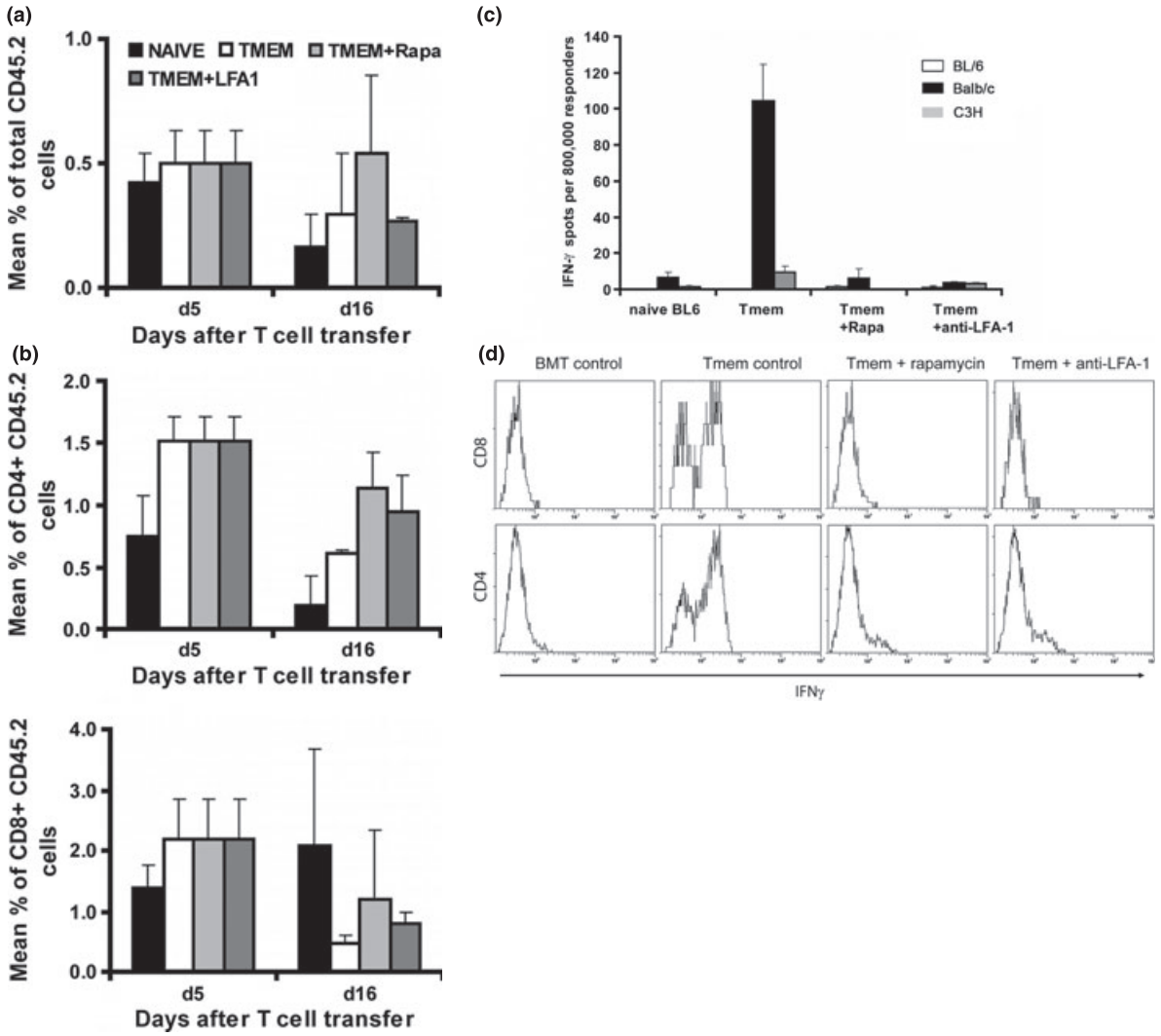

Figure 5 Anti-LFA-1 and rapamycin treatment has no detectable influence on proliferation and survival of transferred memory cells after BMT and leads to abrogation of effector function of transferred Tmem. There was no statistically significant difference regarding proliferation and survival of transferred cells between Tmem recipients treated with either anti-LFA-1 or rapamycin or without additional treatment (pretreatment d5 post-transfer/d-2 post-BMT: $n = 7$ naïve T cells, $n = 19$ Tmem-enriched; 16 days post-transfer/9 days post-BMT: $n = 4$ naïve T cells, $n = 2$ Tmem-enriched, $n = 4$ anti-LFA-1, $n = 3$ rapamycin) in total (a) CD45.2 cells and (b) T cell subpopulations. (c) IFNγ specific ELISPOT analysis shows enhanced donor-specific memory responses in Tmem-enriched recipients which was abrogated in BMT recipients treated with anti-LFA-1 or rapamycin (and in BMT controls; 8 weeks post-BMT, $n = 2$ in all groups). Responses against B6 (white), Balb/c (black), and C3H/N (gray) stimulators are shown. (d) Representative histograms showing donor-specific IFNγ responses in $CD8^+$ (upper panel) and $CD4^+$ (lower panel) lymphocytes (8 weeks post-BMT).

that either relevant proliferation of transferred Tmem cells or rapid elimination occurred in rapamycin or anti-LFA-1 treated Tmem-enriched BMT recipients. More extensive analyses are required, however, to definitively define the kinetics of transferred CD4 and CD8 cells in detail.

Anti-donor memory T cell response is eliminated through rapamycin or anti-LFA-1

To evaluate whether memory T cell responses are in fact attenuated through rapamycin and or anti-LFA-1 post-transplant, IFN-γ production was analyzed in T cells taken from BMT recipients upon *ex vivo* stimulation with donor antigen (8 weeks post-BMT). In ELISPOT assays, Tmem-enriched BMT recipients showed high levels of IFN-γ secretion in response to donor, but not third-party antigen. In contrast, IFN-γ secretion in Tmem-enriched recipients treated with rapamycin or anti-LFA-1 was significantly lower and comparable to naive control animals (Fig. 5c). As ELISPOT assays mainly assess CD8 Tmem reactivity, we also evaluated IFN-γ production by intracellular FACS staining. Here, both $CD4^+$ and $CD8^+$ T cells of

Tmem-enriched BMT recipients produced substantially more IFN-γ than BMT controls. Both rapamycin and anti-LFA-1 drastically reduced production of IFN-γ in both CD4 and CD8 cells (Fig. 5d). Together, these results indicate that the enhanced donor-specific CD4 and CD8 T memory cell response seen in Tmem-enriched BMT recipients becomes virtually eliminated through rapamycin or anti-LFA-1 by 8 weeks post-BMT.

Tolerant chimeras treated with anti-LFA-1 and rapamycin show peripheral and central deletion of donor-reactive T cells

As clonal deletion is a major mechanism of most chimerism protocols, we determined if donor-reactive T cells are deleted in Tmem-enriched recipients. The frequency of certain superantigen-reactive T cell populations corresponds to the deletion of 'truly reactive' donor-specific T cells (as assessed by T cells with a donor-reactive transgenic TCR [39]) and was thus used as a surrogate marker. Developing thymocytes whose TCRs contain Vβ11 and Vβ5 bind to superantigens presented by I-E, and are deleted in Balb/c which are I-E positive mice, but not in B6 mice, which do not express I-E [40–42]. Deletion of CD4 cells was noted in peripheral blood as early as 2 weeks post-BMT in Tmem-enriched mice receiving anti-LFA-1 (data not shown). Six weeks post-BMT, substantial deletion of $CD4^+$ Vβ11 and Vβ5 (but not control Vβ8) cells in peripheral blood was evident in anti-LFA-1 and rapamycin-treated recipients in comparison to naïve B6 mice (and to Tmem control mice) (Fig. 6a). At such early time points, clonal deletion of CD4 (but not CD8) T cells was also observed in the spleen of mice treated with anti-LFA-1 or rapamycin (8 weeks post-BMT) (Fig. 6b). Deletion of Vβ5/Vβ11 $CD4^+$ single positive thymocytes was also evident in anti-LFA-1 and rapamycin-treated animals at this time point (but not in Tmem controls), demonstrating that central clonal deletion occurs in these mice (Fig. 6c). Moreover, deletion of $CD8^+$ splenocytes became detectable late after BMT (20 weeks post-BMT, data not shown). As CD8 T cells – in contrast with CD4 cells – are not deleted extrathymically (as they do not efficiently bind to the superantigen-presenting MHC II), but only intrathymically at the double positive stage of development, this deletion observed among CD8 splenocytes provides additional evidence for central clonal deletion in these chimeras. Taken together, these data suggest peripheral and central clonal deletion of donor-reactive T cells in Tmem-enriched BMT recipients treated with anti-LFA-1 or rapamycin.

Discussion

Overcoming the barrier of Tmem activation remains a considerable challenge when tolerance protocols are applied to nonhuman primates or transplant patients. The murine model developed in the current study was designed to allow the evaluation of treatments for their efficacy to control the Tmem response in recipients of mixed chimerism regimens.

While TCR-transgenic systems facilitate detailed mechanistic studies [16], the described Tmem-enriched model offers the advantage that recipients contain a polyclonal repertoire of Tmem which presumably encompasses a broad spectrum of affinities and specificities, as is usually the case in the clinical setting. Moreover, both CD4 and CD8 Tmem contribute to the elicited rejection in the current model. Interestingly, another study identified virus-induced alloreactive $CD8^+$ central memory T cells to be primarily responsible for memory-mediated rejection [22], whereas in our model, transfer of $CD8^+$ cells alone did not abrogate chimerism (at least not at the cell doses tested). Titrating the number of Tmem that is transferred revealed that certain frequencies of Tmem can be sufficiently controlled through costimulation blockade allowing chimerism to be induced (Fig. 2a). Once a threshold is crossed, however – in our model $2–3 \times 10^7$ transferred T cells from sensitized mice – donor BM is rejected despite costimulation blockade. This chimerism-abrogating effect is antigen-specific as the transfer of equal numbers of T cells from mice sensitized to an unrelated third party skin donor did not prevent chimerism induction (Fig. 1d). These results are consistent with the empirical observation that costimulation blockade-based immunosuppressive therapy is partially, but not completely, effective in nonhuman primates and in renal transplant patients (that can be expected to harbor alloreactive Tmem) [43–46].

A number of selected drugs that we screened in this model failed to overcome the sensitization barrier. Neither bortezomib, anti-TNFα (infliximab) nor anti-IL7 – all with reported beneficial effects on Tmem in other settings [31–35] – were effective as adjunctive treatments in our model (data not shown). However, two drugs – anti-LFA-1 and rapamycin, both approved for clinical application were identified to control Tmem-triggered rejection allowing chimerism and tolerance induction in Tmem-enriched recipients. Indeed, anti-LFA-1 and rapamycin were revealed to abrogate the enhanced responses in both CD4 and CD8 T memory cell subsets. LFA-1, a β2 integrin composed of a unique α-chain (CD11a) noncovalently linked to a β-chain (CD18), has an important role in T cell adhesion and activation and is upregulated on Tmem [47]. Anti-LFA-1 on its own prolongs heart and islet, but not skin allografts and acts synergistically with anti-CD40L, CTLA4Ig, and mTOR inhibitors in various experimental transplant models [48,49]. Notably, CD8 Tmem responses not inhibited by costimulation blockade are susceptible to anti-LFA-1 treatment [50,51]. Recently, an anti-LFA mAb – used in

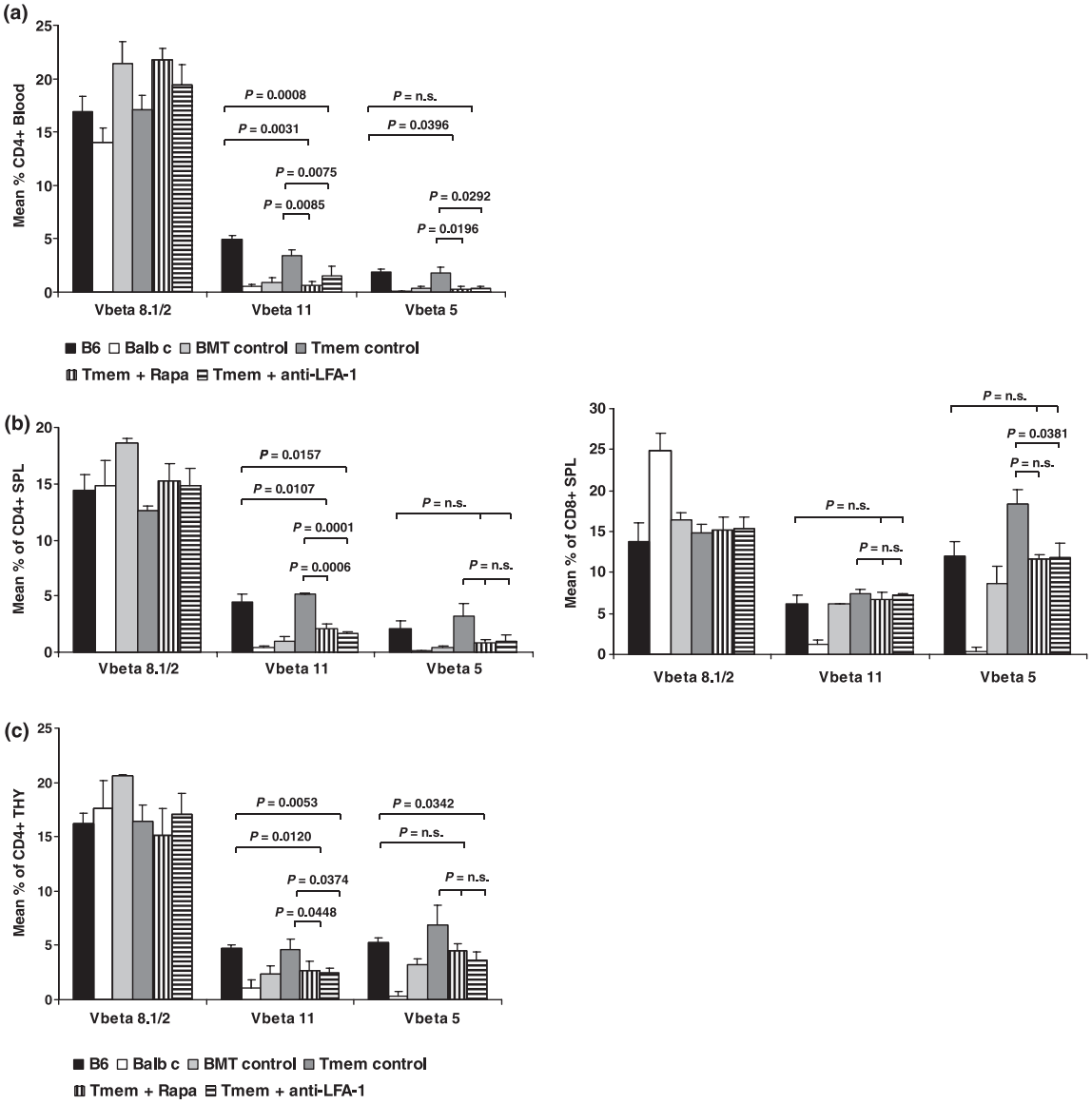

Figure 6 Tolerant chimeras treated with anti-LFA-1 and rapamycin show peripheral and central deletion of donor-reactive T cells. (a) The frequencies of Vβ11 and Vβ5 $CD4^+$ cells (isolated from peripheral blood) were significantly reduced in anti-LFA-1 and rapamycin-treated Tmem-enriched BMT recipients (compared with naïve B6) at 6 weeks post-BMT, suggesting peripheral clonal deletion of donor-reactive CD4 T cells (anti-LFA-1 $n = 7$, rapamycin $n = 12$, Tmem control = 3, BMT control $n = 6$, naïve Balb/C $n = 3$, and naive B6 $n = 3$). (b) The frequencies of Vβ11 and Vβ5 among $CD4^+$ and among $CD8^+$ splenocytes were measured at 8 weeks post-BMT (anti-LFA-1 $n = 3$, rapamycin $n = 3$, Tmem control = 3, BMT control $n = 3$, naïve Balb/C $n = 3$, and naive B6 $n = 3$). Significantly lower levels of $CD4^+$ Vβ11 cells (and numerically lower levels of $CD4^+$ Vβ5 cells) were evident in anti-LFA-1 and rapamycin-treated groups (compared with naïve B6). No such deletion was observed at this early time point among $CD8^+$ cells, suggesting that the deletion of CD4 T cells early post-BMT took place extrathymically (8 weeks post-BMT; anti-LFA-1 $n = 3$, rapamycin $n = 3$, Tmem control = 3, BMT control $n = 3$, naïve Balb/C $n = 3$, and naive B6 $n = 3$). (c) Flow cytometric analysis of thymocytes revealed reduced frequencies of Vβ11 and Vβ5 among $CD4^+$ single positive cells in anti-LFA-1 ($n = 3$) and rapamycin-treated recipients ($n = 3$) in comparison to naïve B6 control mice ($n = 3$), indicating central clonal deletion of donor-reactive thymocytes (Tmem control $n = 3$, BMT control $n = 3$ and naïve Balb/C $n = 3$). (Panel a–c) Naïve B6 are shown in black, naïve Balb/c in white, BMT control in light-gray, control Tmem-enriched recipients in dark-gray, Tmem-enriched recipients treated with anti-LFA-1 in white with black vertical stripes, Tmem-enriched recipients treated with rapamycin in white with black horizontal stripes.

combination with belatacept, a second generation CTLA4Ig – was demonstrated to prolong islet allograft survival in a nonhuman primate model by controlling the Tmem response [52]. A humanized anti-CD11a mAb (efalizumab) was approved for treatment of severe psoriasis [53] and has been evaluated in renal [54] and in islet transplant recipi-

ents [55]. While efalizumab was recently withdrawn by the manufacturer because of the occurrence of progressive multi-focal leukoencephalopathy in rare cases, this devastating complication occurred with long-term use. Thus, short-term therapy with efalizumab for appropriate indications might still be considered acceptably safe.

Although underlying mechanisms still need to be clarified, the engraftment enhancing effect seen with anti-LFA-1 might be due altered Tmem trafficking impairing rejection. Besides, anti-LFA-1 might affect Treg function. Recent data suggest that treatment with anti-LFA-1 and CTLA4-Ig leads to a selective enrichment of Tregs ($CD4^+CD25^+FoxP3^+$) in peripheral lymph nodes in a fully allogeneic murine skin transplantation model [56]. Simultaneously, within the same model, activated effector cells were found to undergo increased apoptosis within lymph nodes. As well, in another allogeneic murine transplant model, anti-LFA-1 was found to promote retention of Tregs in lymph nodes, as well as inhibition of cytokine production in Tmem cells [57].

Inhibition of mTOR (with rapamycin or its derivatives) leads to a series of effects that is considerably more complex than has been initially recognized [58]. In particular, mTOR inhibition enhances the Tmem responses to viral infections [59], but not the Tmem response to transplant antigens [60]. mTOR inhibition has also been noted to promote regulatory T cell generation and function [61]. In the context of chimerism induction in naïve recipients, mTOR inhibitors promote engraftment of allogeneic BM by themselves [7,49,62] and in synergy with regulatory T cell therapy [63]. While rapamycin failed to promote tolerance to anti-donor memory cells in a previous study [22], the current results extend the beneficial effects of mTOR inhibition in BMT recipients to those that contain a substantial frequency of donor-reactive Tmem. This discrepancy may be because of the different dosing regimens of rapamycin. The therapeutic effect of rapamycin on alloreactive memory T cell responses observed in the current experiments is in line with recent reports on the distinct effects of mTOR inhibition on this Tmem subset [59,60]. Another possible mechanism of rapamycin in this model might be its effect on Treg function. Rapamycin has already been shown to synergize with therapeutic Treg treatment to enhance BM engraftment in this model [63]. Moreover, recent findings demonstrated that rapamycin increased suppressive capacity of Tregs in a NHP *in vitro* model [64].

Tmem-mediated rejection leading to graft failure is of concern in clinical BMT for conventional indications, in particular in recipients treated with reduced intensity conditioning [65,66]. Notably, rapamycin is under clinical investigation for graft-versus-host-disease prophylaxis [67]. Our results suggest that the effect of rapamycin on Tmem activation might be beneficial in a wide range of BMT recipients and deserves further investigation.

The model presented herein is expected to enhance the predictive value of murine mixed chimerism studies and should be helpful in the development of clinically viable tolerance protocols. Anti-LFA-1 and rapamycin have been identified as drugs with efficacy in controlling Tmem in recipients of nonmyeloablative BMT and are attractive candidates for evaluation in preclinical and clinical mixed chimerism studies.

Authorship

HR, NP: designed and performed research, analyzed data, wrote paper. CK, LU, CS, UB, MG, KH, ES and FW: performed research. TW: designed research, analyzed data, wrote paper.

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References

1. Pilat N, Wekerle T. Transplantation tolerance through mixed chimerism. *Nat Rev Nephrol* 2010; **6**: 594.
2. Kawai T, Cosimi AB, Spitzer TR, *et al.* HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med* 2008; **358**: 353.
3. Scandling JD, Busque S, Dejbakhsh-Jones S, *et al.* Tolerance and chimerism after renal and hematopoietic-cell transplantation. *N Engl J Med* 2008; **358**: 362.
4. Kean LS, Gangappa S, Pearson TC, Larsen CP. Transplant tolerance in non-human primates: progress, current challenges and unmet needs. *Am J Transplant* 2006; **6**: 884.
5. Wekerle T, Kurtz J, Ito H, *et al.* Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. *Nat Med* 2000; **6**: 464.
6. Durham MM, Bingaman AW, Adams AB, *et al.* Administration of anti-CD40 ligand and donor bone marrow leads to hematopoietic chimerism and donor-specific tolerance without cytoreductive conditioning. *J Immunol* 2000; **165**: 1.
7. Blaha P, Bigenzahn S, Koporc Z, *et al.* The influence of immunosuppressive drugs on tolerance induction through bone marrow transplantation with costimulation blockade. *Blood* 2003; **101**: 2886.

8. Taylor PA, Lees CJ, Waldmann H, Noelle RJ, Blazar BR. Requirements for the promotion of allogeneic engraftment by anti-CD154 (anti-CD40L) monoclonal antibody under nonmyeloablative conditions. *Blood* 2001; **98**: 467.
9. Takeuchi Y, Ito H, Kurtz J, Wekerle T, Ho L, Sykes M. Earlier low-dose TBI or DST overcomes $CD8^+$ T-cell-mediated alloresistance to allogeneic marrow in recipients of anti-CD40L. *Am J Transplant* 2004; **4**: 31.
10. Sykes M. Hematopoietic cell transplantation for tolerance induction: animal models to clinical trials. *Transplantation* 2009; **87**: 309.
11. Ochiai T, Benichou G, Cosimi AB, Kawai T. Induction of allograft tolerance in nonhuman primates and humans. *Front Biosci* 2007; **12**: 4248.
12. Wekerle T, Sayegh MH, Hill J, *et al.* Extrathymic T cell deletion and allogeneic stem cell engraftment induced with costimulatory blockade is followed by central T cell tolerance. *J Exp Med* 1998; **187**: 2037.
13. Adams AB, Durham MM, Kean L, *et al.* Costimulation blockade, busulfan, and bone marrow promote titratable macrochimerism, induce transplantation tolerance, and correct genetic hemoglobinopathies with minimal myelosuppression. *J Immunol* 2001; **167**: 1103.
14. Wekerle T, Kurtz J, Bigenzahn S, Takeuchi Y, Sykes M. Mechanisms of transplant tolerance induction using costimulatory blockade. *Curr Opin Immunol* 2002; **14**: 592.
15. Kawai T, Sogawa H, Boskovic S, *et al.* CD154 blockade for induction of mixed chimerism and prolonged renal allograft survival in nonhuman primates. *Am J Transpl* 2004; **4**: 1391.
16. Larsen CP, Page A, Linzie KH, *et al.* An MHC-defined primate model reveals significant rejection of bone marrow after mixed chimerism induction despite full MHC matching. *Am J Transplant* 2010; **10**: 2396.
17. Yang YG, deGoma E, Ohdan H, *et al.* Tolerization of anti-Gala1-3Gal natural antibody-forming B cells by induction of mixed chimerism. *The J Exp Med* 1998; **187**: 1335.
18. Porcheray F, Wong W, Saidman SL, *et al.* B-cell immunity in the context of T-cell tolerance after combined kidney and bone marrow transplantation in humans. *Am J Transplant* 2009; **9**: 2126.
19. Valujskikh A, Baldwin WM 3rd, Fairchild RL. Recent progress and new perspectives in studying T cell responses to allografts. *Am J Transplant* 2010; **10**: 1117.
20. Page AJ, Ford ML, Kirk AD. Memory T-cell-specific therapeutics in organ transplantation. *Curr Opin Organ Transplant* 2009; **14**: 643.
21. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* 2004; **22**: 745.
22. Adams AB, Williams MA, Jones TR, *et al.* Heterologous immunity provides a potent barrier to transplantation tolerance. *J Clin Invest* 2003; **111**: 1887.
23. Wu Z, Bensinger SJ, Zhang J, *et al.* Homeostatic proliferation is a barrier to transplantation tolerance. *Nat Med* 2004; **10**: 87.
24. Colson YL, Schuchert MJ, Ildstad ST. The abrogation of allosensitization following the induction of mixed allogeneic chimerism. *J Immunol* 2000: **165**; 637.
25. Nagata S, Okano S, Yonemitsu Y, *et al.* Critical roles of memory T cells and antidonor immunoglobulin in rejection of allogeneic bone marrow cells in sensitized recipient mice. *Transplantation* 2006; **82**: 689.
26. Heeger PS. T-cell allorecognition and transplant rejection: a summary and update. *Am J Transplant* 2003; **3**: 525.
27. Valujskikh A, Pantenburg B, Heeger PS. Primed allospecific T cells prevent the effects of costimulatory blockade on prolonged cardiac allograft survival in mice. *Am J Transplant* 2002; **2**: 501.
28. Stapler D, Lee ED, Selvaraj SA, *et al.* Expansion of effector memory TCR $Vbeta4^+$ $CD8^+$ T cells is associated with latent infection-mediated resistance to transplantation tolerance. *J Immunol* 2008; **180**: 3190.
29. Blaha P, Bigenzahn S, Koporc Z, Sykes M, Muehlbacher F, Wekerle T. Short-term immunosuppression facilitates induction of mixed chimerism and tolerance after bone marrow transplantation without cytoreductive conditioning. *Transplantation* 2005; **80**: 237.
30. Nierlich PN, Klaus C, Bigenzahn S, *et al.* The role of natural killer T cells in costimulation blockade-based mixed chimerism. *Transpl Int* 2010; **23**: 1179.
31. Kim JS, Lee JI, Shin JY, *et al.* Bortezomib can suppress activation of rapamycin-resistant memory T cells without affecting regulatory T-cell viability in non-human primates. *Transplantation* 2009; **88**: 1349.
32. Wollin M, Abele S, Bruns H, *et al.* Inhibition of TNF-alpha reduces transplant arteriosclerosis in a murine aortic transplant model. *Transpl Int* 2009; **22**: 342.
33. Jacobsohn DA. Emerging therapies for graft-versus-host disease. *Expert Opin Emerg Drugs* 2003; **8**: 323.
34. Bruns H, Meinken C, Schauenberg P, *et al.* Anti-TNF immunotherapy reduces $CD8^+$ T cell-mediated antimicrobial activity against Mycobacterium tuberculosis in humans. *J Clin Invest* 2009; **119**: 1167.
35. Wang Y, Dai H, Liu Z, Cheng X, Tellides G, Dai Z. Neutralizing IL-7 promotes long-term allograft survival induced by CD40/CD40L costimulatory blockade. *Am J Transplant* 2006; **6**: 2851.
36. Bucy RP, Li J, Huang GQ, Honjo K, Xu XY. Allograft tolerance induced by combined anti-LFA-1 and anti-ICAM-1 mAb is associated with shift from Th1 to Th2 cytokine expression in allograft. *FASEB J* 1995; **9**: A497.
37. Corbascio M, Ekstrand H, Osterholm C, *et al.* CTLA4Ig combined with anti-LFA-1 prolongs cardiac allograft survival indefinitely. *Transpl Immunol* 2002; **10**: 55.
38. Thomson AW, Turnquist HR, Raimondi G. Immunoregulatory functions of mTOR inhibition. *Nat Rev Immunol* 2009; **9**: 324.
39. Kurtz J, Shaffer J, Lie A, Anosova N, Benichou G, Sykes M. Mechanisms of early peripheral CD4 T cell tolerance induction by anti-CD154 monoclonal antibody and allogeneic

bone marrow transplantation: evidence for anergy and deletion, but not regulatory cells. *Blood* 2004; **103**: 4336.
40. Tomonari K, Fairchild S. The genetic basis of negative selection of Tcrb-V11+ T cells. *Immunogenetics* 1991; **33**: 157.
41. Bill J, Kanagawa O, Woodland D, Palmer E. The MHC molecule I-E is necessary but not sufficient for the clonal deletion of VB11 bearing T cells. *J Exp Med* 1989; **169**: 1405.
42. Dyson PJ, Knight AM, Fairchild S, Simpson E, Tomonari K. Genes encoding ligands for deletion of Vb11 T cells cosegregate with mammary tumour virus genomes. *Nature* 1991; **349**: 531.
43. Kirk AD, Harlan DM, Armstrong NN, *et al.* CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc Natl Acad Sci USA* 1997; **94**: 8789.
44. Larsen CP, Pearson TC, Adams AB, *et al.* Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am J Transplant* 2005; **5**: 443.
45. Vincenti F, Larsen C, Durrbach A, *et al.* Costimulation blockade with belatacept in renal transplantation. *N Engl J Med* 2005; **353**: 770.
46. Weaver TA, Charafeddine AH, Agarwal A, *et al.* Alefacept promotes co-stimulation blockade based allograft survival in nonhuman primates. *Nat Med* 2009; **15**: 746.
47. Ford ML, Larsen CP. Translating costimulation blockade to the clinic: lessons learned from three pathways. *Immunol Rev* 2009; **229**: 294.
48. Nicolls MR, Gill RG. LFA-1 (CD11a) as a therapeutic target. *Am J Transplant* 2006; **6**: 27.
49. Metzler B, Gfeller P, Bigaud M, *et al.* 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. *J Immunol* 2004; **173**: 7025.
50. Kitchens WH, Haridas D, Wagener ME, *et al.* Integrin antagonists prevent costimulatory blockade-resistant transplant rejection by CD8(+) memory T cells. *Am J Transplant* 2011; **12**: 69.
51. Setoguchi K, Schenk AD, Ishii D, *et al.* LFA-1 antagonism inhibits early infiltration of endogenous memory CD8 T cells into cardiac allografts and donor-reactive T cell priming. *Am J Transplant* 2011; **11**: 923.
52. Badell IR, Russell MC, Thompson PW, *et al.* LFA-1-specific therapy prolongs allograft survival in rhesus macaques. *J Clin Invest* 2010; **120**: 4520.
53. Lebwohl M, Tyring SK, Hamilton TK, *et al.* A novel targeted T-cell modulator, efalizumab, for plaque psoriasis. *N Engl J Med* 2003; **349**: 2004.
54. Vincenti F, Mendez R, Pescovitz M, *et al.* A phase I/II randomized open-label multicenter trial of efalizumab, a humanized anti-CD11a, anti-LFA-1 in renal transplantation. *Am J Transplant* 2007; **7**: 1770.
55. Turgeon NA, Avila JG, Cano JA, *et al.* Experience with a novel efalizumab-based immunosuppressive regimen to facilitate single donor islet cell transplantation. *Am J Transplant* 2010; **10**: 2082.
56. Reisman NM, Floyd TL, Wagener ME, Kirk AD, Larsen CP, Ford ML. LFA-1 blockade induces effector and regulatory T-cell enrichment in lymph nodes and synergizes with CTLA-4Ig to inhibit effector function. *Blood* 2011; **118**: 5851.
57. Kitchens WH, Haridas D, Wagener ME, Song M, Ford ML. Combined costimulatory and leukocyte functional antigen-1 blockade prevents transplant rejection mediated by heterologous immune memory alloresponses. *Transplantation* 2012; **93**: 997.
58. Thomson AW, Knolle PA. Antigen-presenting cell function in the tolerogenic liver environment. *Nat Rev* 2010; **10**: 753.
59. Araki K, Turner AP, Shaffer VO, *et al.* mTOR regulates memory CD8 T-cell differentiation. *Nature* 2009; **460**: 108.
60. Ferrer IR, Wagener ME, Robertson JM, *et al.* Cutting edge: rapamycin augments pathogen-specific but not graft-reactive $CD8^+$ T cell responses. *J Immunol* 2010; **185**: 2004.
61. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands $CD4^+CD25^+FoxP3^+$ regulatory T cells. *Blood* 2005; **105**: 4743.
62. Taylor PA, Lees CJ, Wilson JM, *et al.* Combined effects of calcineurin inhibitors or sirolimus with anti-CD40L mAb on alloengraftment under nonmyeloablative conditions. *Blood* 2002; **100**: 3400.
63. Pilat N, Baranyi U, Klaus C, *et al.* Treg-therapy allows mixed chimerism and transplantation tolerance without cytoreductive conditioning. *Am J Transplant* 2010; **10**: 751.
64. Singh K, Kozyr N, Stempora L, *et al.* Regulatory T cells exhibit decreased proliferation but enhanced suppression after pulsing with sirolimus. *Am J Transplant* 2012; **12**: 1441.
65. Komatsu M, Mammolenti M, Jones M, Jurecic R, Sayers TJ, Levy RB. Antigen-primed $CD8^+$ T cells can mediate resistance, preventing allogeneic marrow engraftment in the simultaneous absence of perforin-, CD95L-, TNFR1-, and TRAIL-dependent killing. *Blood* 2003; **101**: 3991.
66. Zimmerman Z, Shatry A, Deyev V, *et al.* Effector cells derived from host CD8 memory T cells mediate rapid resistance against minor histocompatibility antigen-mismatched allogeneic marrow grafts without participation of perforin, Fas ligand, and the simultaneous inhibition of 3 tumor necrosis factor family effector pathways. *Biol Blood Marrow Transplant* 2005; **11**: 576.
67. Cutler C, Kim HT, Hochberg E, *et al.* Sirolimus and tacrolimus without methotrexate as graft-versus-host disease prophylaxis after matched related donor peripheral blood stem cell transplantation. *Biol Blood Marrow Transplant* 2004; **10**: 328.