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Research Article

MHC Disparate Resting B Cells Are Tolerogenic in the Absence of Alloantigen-Expressing Dendritic Cells

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Resting B cell (rB) populations have been shown to tolerize to soluble proteins and to minor-H but not to MHC alloantigens. We speculated that the reason for failing to tolerize to MHC alloantigen is that the few remaining dendritic cells (DCs) contaminating purified rB cell populations efficiently activate MHC allospecific T cells which are present at a higher frequency than T cells specific for minor-H alloantigen and soluble proteins. We established that MHC disparate rB cells are indeed tolerogenic when devoid of DC populations, as parental strain mice showed delayed D^d skin graft rejection when infused with rB cells from mice in which MHC class I D^d alloantigen was specifically targeted to T and B cells (CD2-D^d transgenic mice). In contrast, treatment of parental strain mice with allogeneic rB cells purified from MHC-D^d transgenic mice, in which D^d is ubiquitously expressed, including DCs, induced accelerated D^d graft rejection. We also showed that adding only 5,000 D^d expressing DCs to CD2-D^d rB cells abrogated the tolerogenic effect. Surprisingly, allogeneic rB cells prolonged graft survival in D^d-primed mice. Thus, MHC disparate rB cells are tolerogenic and their failure to delay graft rejection can be explained by contaminating allogeneic DCs.

1. Introduction

Resting B cells have been suggested to be potent inducers of specific unresponsiveness both *in vitro* and *in vivo* [1, 2] and have been thought to mediate such effects by directly presenting Ag (signal 1) in the absence of productive costimulatory interactions, principally mediated by B7-CD28 and CD40-CD40L interactions (signal 2) [3–6]. This hypothesis, however, has been challenged by studies demonstrating that rB cells not only express CD40, but also upregulate expression of B7 family members in a timely fashion *in vivo* [7]. Moreover, it has become increasingly appreciated that indirect antigen presentation, in which antigen shed from donor cells is presented via immature host DC, may be crucial for tolerance induction [8–11]. Regardless of the mechanism, rB cells clearly have tolerogenic effects on T cells specific for some types of antigen, but not others. For instance, rB cells induce tolerance to soluble proteins [2, 12] and to minor-H mismatched transplants [1], but not to transplants discrepant in MHC alloantigens [7].

That MHC disparate grafts present a higher bar for tolerance induction in general is emphasized by studies showing that MHC-expressing allografts are rejected even in the absence of the "danger" signals that appear requisite in generation of immune responses to other antigens, presumably by enhancement of APC activity [13]. One explanation for the ability of rB cells to induce tolerance to minor-H but not MHC disparate grafts is that the few DCs associated with rB cells purified from normal mice effectively activate the far more frequent and high affinity MHC allospecific T cells. To investigate this possibility, we assessed the ability of allogeneic rB cells, in which alloantigen expression is genetically restricted to T and B cells, to thwart allograft rejection.

2. Results

2.1. Allogeneic rB Cells from Gene-Targeted Mice Induce Hyporesponsiveness. To assess the effects of rB cell presentation of

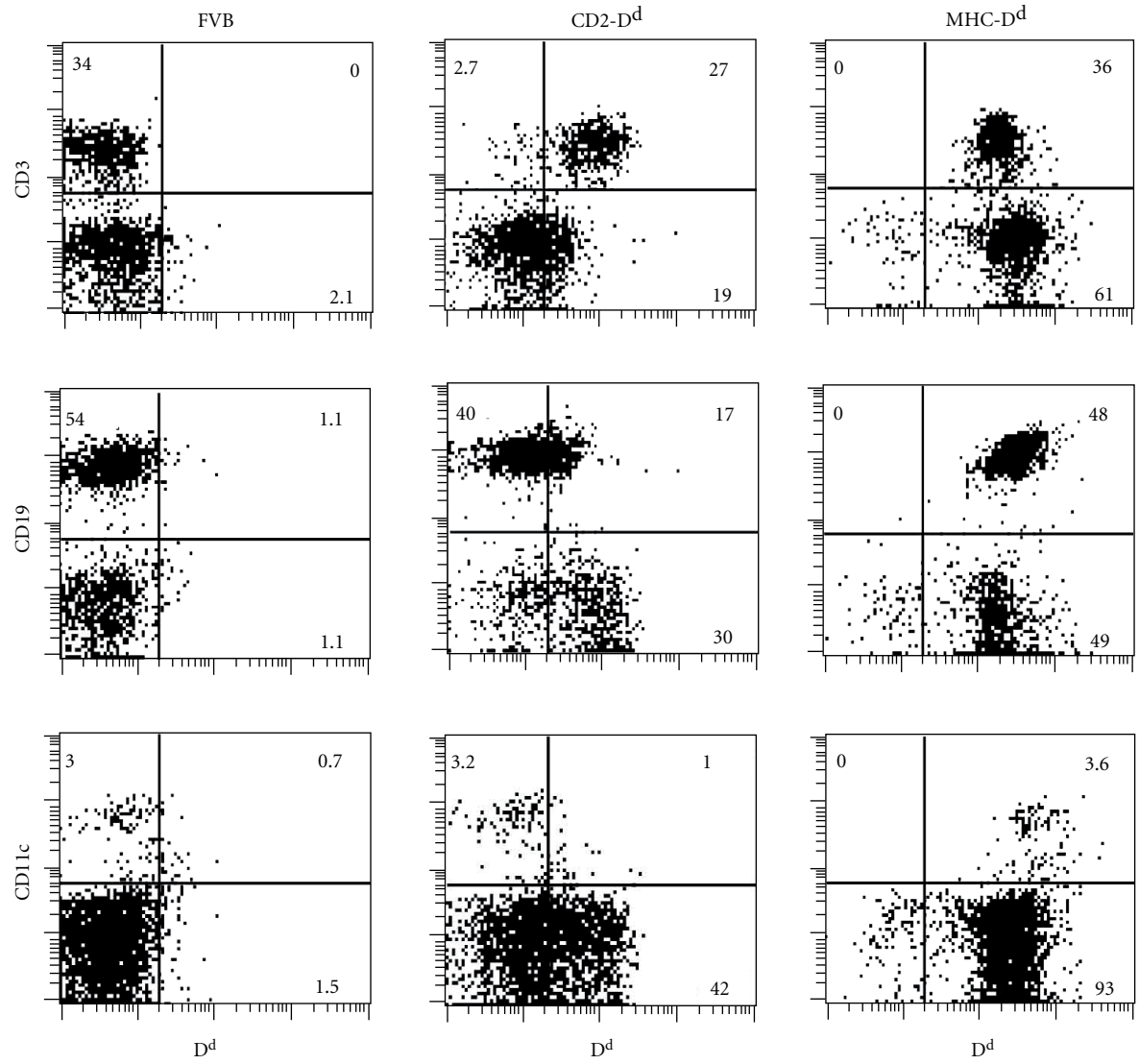

Figure 1: D^d expression on splenic T cells, B cells, and DC from FVB, MHC-D^d, and CD2-D^d transgenic mice.

class I MHC alloantigen on rejection responses *in vivo*, we utilized lymphocyte populations from two lines of transgenic mice, because of their differential expression of D^d alloantigen on cellular populations. D^d is ubiquitously expressed in MHC-D^d transgenic mice (FVB background, H-2^q), with DC expressing low to moderate levels, while in CD2-D^d transgenic mice, (FVB)D^d is expressed on T and B cells, but not on $CD11c^+$ DC [14] (Figure 1). rB cells recovered from the spleens of the two transgenic lines after extensive depletion of T cells, macrophages, and DC populations were comparable with respect to DC content (<1%), as well as expression of B lineage and costimulatory markers (Figure 2). Naive and D^d-primed FVB mice were pretreated with 10^7rB cells from MHC-D^d or CD2-D^d mice. Though B cells purified from these two transgenics were highly comparable in terms of DC content and other markers (Figure 2), they induced dramatically different effects on D^d graft rejection when infused into host FVB mice: MHC-D^d B cells induced accelerated graft rejection median survival time (MST) 9 days versus 12 days for control mice, whereas CD2-D^d B cells caused delayed graft rejection (MST 18 days) (Figure 3(a)). Surprisingly, infusing either B cell population into primed mice consistently abrogated accelerated rejection (MST 12 days versus 8.5 days for control) and converted response kinetics to a primary rejection rate (Figure 3(b)). Thus, MHC disparate rB cells from gene-targeted mice induced hyporesponsiveness in both naïve and memory allospecific $CD8^+$ T cells while paradoxically, MHC D^d rB cells containing small numbers of alloantigen-expressing DC, amidst a plethora of rB cells, efficiently primed naïve $CD8^+$ T cells, but failed to significantly activate memory class I allospecific T cells.

Similar results were observed in naïve thymectomized mice infused with CD2-D^d cells (MST 17 days versus 12 days for control) or MHC-D^d B (MST 8 days), indicating

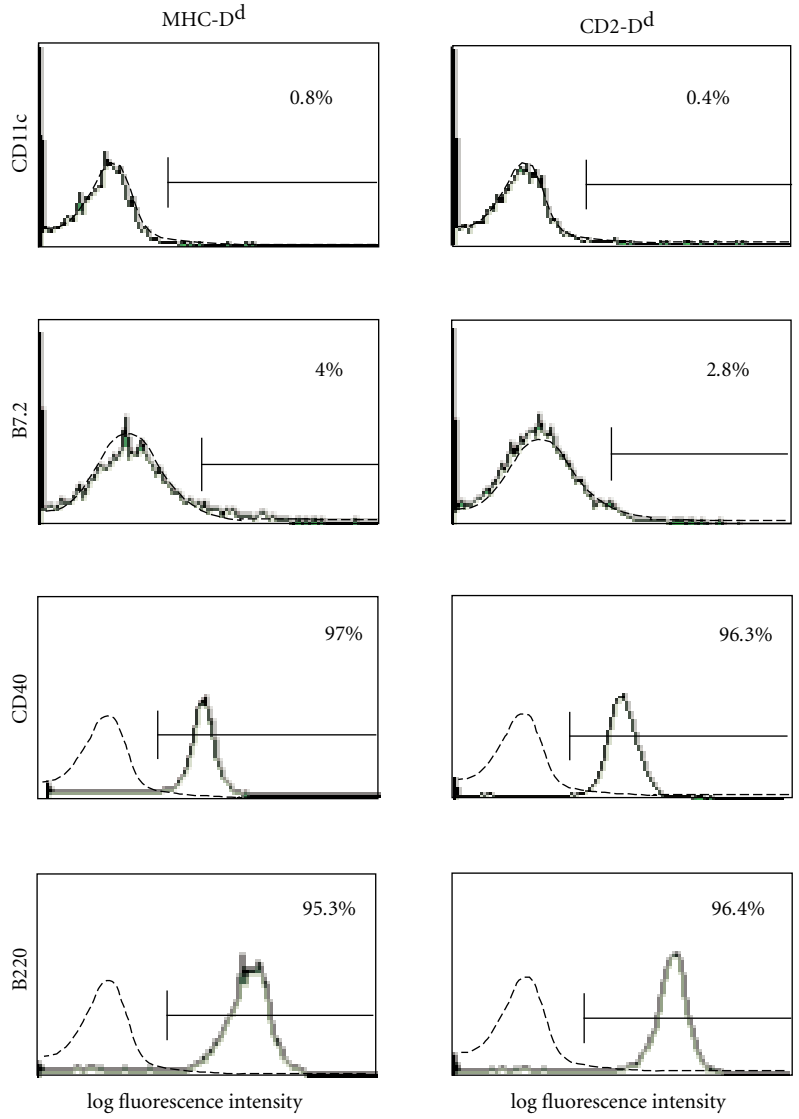

Figure 2: B cell profiles after depletion of T cell, macrophages, and DC from spleen cells of MHC-D^d and CD2-D^d transgenic mice. Isotype-matched controls are shown with dotted lines.

that rejection did not depend on the activity of lymphocytes newly emigrated from the thymus that had not been initially exposed to alloantigen. Furthermore, we do not think that the difference in response is attributable to higher D^d expression on MHC-D^d versus CD2-D^d B cells because in our CD2-D^d transgenic mouse lines, with line-dependent D^d expression levels, we have observed that tolerance, as assessed by skin allograft rejection, correlated directly with the D^d expression level [14], suggesting that extensive TCR cross-linking by the rB cells is of importance in tolerance induction [15]. These data suggest that if D^d expression was as high on CD2-D^d B cell as on MHC-D^d rB cells, the CD2-D^d cells would be able to induce more prolonged hyporesponsiveness, or even tolerance.

2.2. Primary and Secondary Graft Rejection Responses Mediated by CD8$^+$ T Cells Are Abrogated by Genetically Targeted D^d-Expressing rB Cells. To better explore the effects of rB cells on the T cells mediating rejection, we utilized an adoptive transfer model in which T-depleted FVB host mice (thymectomized and irradiated with 500 cGy [16]) were reconstituted with limiting numbers of CD8$^+$ T responder cells. CD8$^+$ T cells are both necessary and sufficient to reject MHC class I disparate D^d skin allografts in naïve mice and for accelerated rejection in antigen primed-mice, whereas CD4$^+$ T cells are neither necessary nor sufficient for allograft rejection [17, 18].

D^d skin allograft rejection was substantially delayed in naïve CD8$^+$ T cell-reconstituted mice that were infused with CD2-D^d rB cells (MST 35.5 days versus 18.5 days) (Figure 4). Even more striking was the substantially prolonged graft survival in mice reconstituted with D^d-primed CD8$^+$ T cells

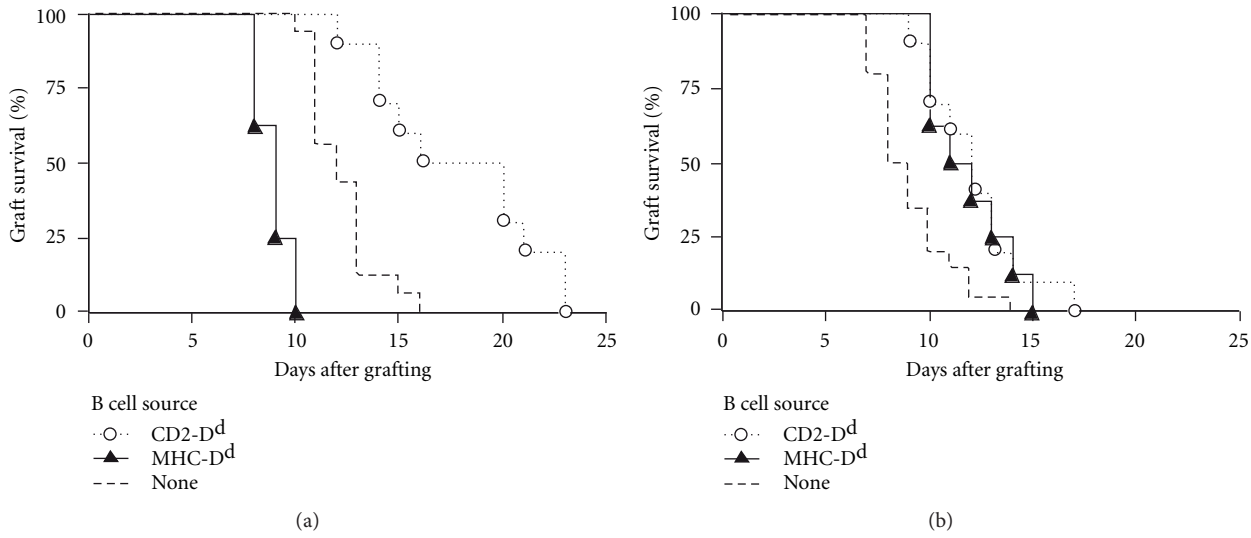

Figure 3: Rejection of D^d skin grafts in mice infused with resting B cells. (a) Naive mice received no injection, 10^7 purified MHC-D^d B cells, or 10^7 purified CD2-D^d B cells intravenously: (filled triangle) = MHC-D^d; (open circle) = CD2-D^d; and the dashed line = none. Mice were grafted with MHC-D^d tail skin 2 weeks later. MST of skin grafts: naive mice = 12 days ($n = 16$); naive plus CD2-D^d B cell = 18 days ($P < 0.001$ versus naive, by log-rank) ($n = 10$); naive plus MHC-D^d B cell = 9 days ($P < 0.001$ versus naive) ($n = 8$). (b) D^d-primed mice received no injection, 10^7 purified MHC-D^d B cells, or 10^7 purified CD2-D^d B cells intravenously: filled triangle = MHC-D^d; open circle = CD2-D^d; and the dashed line = none. Mice were grafted with MHC-D^d tail skin 2 wks later. MST of skin grafts: primed mice = 8.5 d ($n = 20$); priming plus CD2-D^d B cell = 12 d ($P = 0.021$ versus primed; $P = 0.87$ versus naive) ($n = 10$); priming plus MHC-D^d B cell = 11.5 days ($P = 0.012$ versus primed; $P = 0.42$ versus naive) ($n = 8$).

and infused with CD2-D^d B cells (MST 17.5 versus 7.5 days) (Figure 4). These data confirm that both naïve and memory $CD8^+$ allospecific T cells are rendered hyporesponsive *in vivo* by alloantigen expressed on nonprofessional APCs, when contaminating allogeneic DCs are eliminated.

2.3. Effects of Number of Dendritic Cells on Priming versus Hyporesponsiveness. Since contaminating DC in purified populations of rB cells are likely responsible for priming naïve class I allospecific lymphocytes, we wished to quantitate the minimum number required. Accordingly, we added graded numbers of MHC-D^d DCs (positively selected by cell sorting with anti-CD11c mAb and identified as 97% pure) to 10^7 purified CD2-D^d B cells and infused the cell mixture into naive FVB mice. Remarkably, as few as 5,000 DCs (0.005%) abrogated the tolerogenic effect of the rB cells, and partially primed naive allospecific T cells with full priming were achieved with as few as 10,000 DCs (Table 1). Clearly, MHC-D^d DC can accelerate D^d skin graft rejection via direct antigen presentation. In contrast, cross-presentation of D^d peptides by DC from CD2-D^d (4906) mice might rather be expected to tolerize to D^d [19], in which case the delayed skin graft rejection observed on infusion of CD2-D^d rB cells could theoretically be attributable to the DC contaminating the CD2-D^d rB cell preparation, rather than to the rB cells themselves. To test this possibility, we purified $CD11c^+$ DC by MACS from FVB, MHC-D^d (3604), and CD2-D^d (4906) mice. Log doses of these cells were transferred i.v. into FVB recipients. Two weeks later, the mice were given D^d (3604) skin grafts. The MHC-D^d DC accelerated graft rejection at the 10^5 and 10^6 DC doses (Figure 5). While DC from the CD2-D^d mice failed to delay graft rejection at any dose tested remarkably, rejection was accelerated at the highest (10^6) DC dose. Thus, the infused DCs from CD2-D^d mice were not responsible for the induction of hyporesponsiveness to allogeneic skin grafts. Moreover, even in genetic targeting strategies in which alloantigen is not directly expressed by DC, but rather may be acquired from neighboring cells, high doses of DC may prime rather than tolerize, an important caveat for this tolerance strategy.

3. Discussion

Our studies indicate that MHC disparate rB cells are tolerogenic, when alloantigen-expressing DCs are completely eliminated, implicating contaminating DC as a source of allostimulation in B lymphocyte populations isolated from normal mice. Thus, in an ideal world, where all potential DC can be depleted, one could anticipate that purified populations of rB cells would be tolerogenic, even for MHC alloantigenic disparities. The likelihood that current technologies can reduce the DC content of a cellular population to <0.005%, the percentage of DCs that gave rise to partial priming for a single class I MHC mismatch is low. A gene therapy approach [20] with B cell-specific transduction of alloantigen may hold more promise in facilitating alloengraftment in human clinical trials. However, our data indicate that the

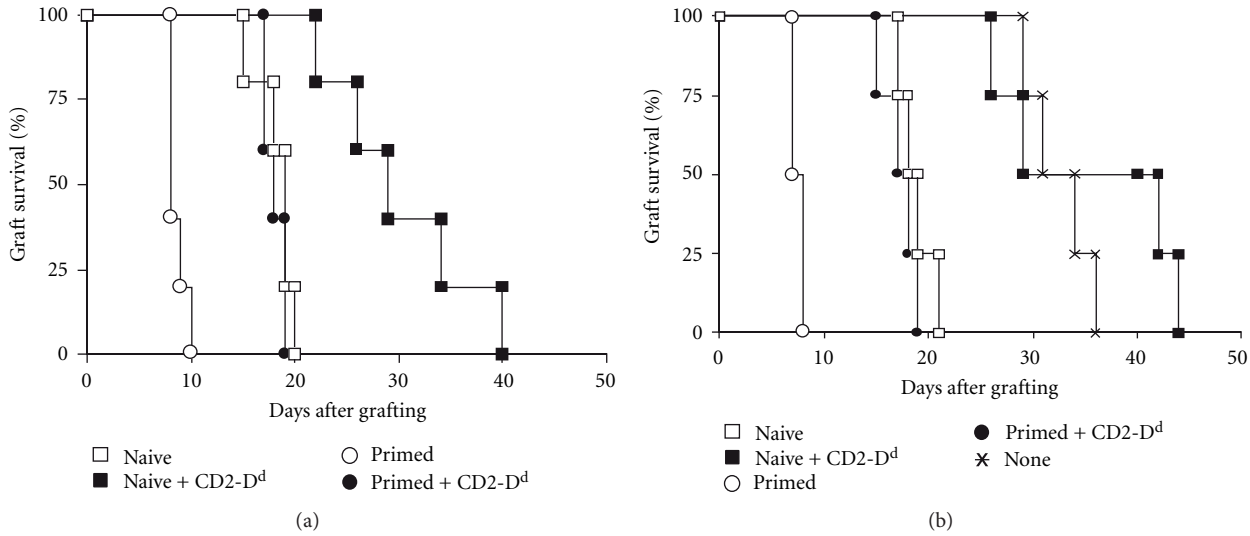

FIGURE 4: Rejection of D^d skin grafts in adoptively transferred mice. T cells (a) or purified $CD8^+$ T cells (b) from D^d primed or naive FVBs were transferred into ATX FVB mice 1 week after irradiation (500 cGy). Mice were infused with CD2-D^d B cells 1 wk after T cell transfer, and were grafted with MHC-D^d tail skin 2 wks later. MST of skin grafts: naive $CD8^+$ T cell-infused mice (open square) = 18.5 days ($n = 4$); naive $CD8^+$ T cell plus CD2-D^d B cell-treated mice (filled square) = 35.5 days ($n = 4$); primed $CD8^+$ T cell plus CD2-D^d B cell-treated mice (filled circle) = 17.5 days ($n = 4$); primed $CD8^+$ T cell-infused mice (open circle) = 7.5 days ($n = 4$); irradiation alone (x) = 32.5 days ($n = 4$).

TABLE 1: Effects of number of dendritic cells (DCs) on priming versus hyporesponsiveness[a].

Dendritic cell dose	Percentage of infused cells	Graft survival (days)	MST (days)
1×10^5	1	8, 8, 9	8
5×10^4	0.5	8, 9, 9	9
1×10^4	0.01	8, 9, 14	9
5×10^3	0.005	13, 17, 23	17
1×10^3	0.0001	21, 22, 22	22
0	0	20, 20, 21	20

[a]Graded numbers of MHC-D^d DCs were added to 1×10^7 purified CD2-D^d B cells and infused into naive FVB mice. After 2 weeks, mice were engrafted with MHC-D^d tail skin grafts and monitored daily for rejection.

success of this approach requires limitations in the numbers of nontargeted DC in the infusate, as expression of allogeneic peptides on nontargeted DC, which acquire allopeptide via cross-presentation mechanisms, provokes immunity.

Quite perplexing was the finding that the memory (accelerated) rejection response, mediated by $CD8^+$ T memory cells, was eliminated by the same population of rB cells that primed naïve $CD8^+$ T cells (i.e., by MHC-D^d rB cells), suggesting that memory $CD8^+$ T cells are even more sensitive than naïve T cells to tolerogenic effects of quiescently presented antigen and require either larger numbers of DC than were provided in purified MHC-D^d B cell populations, or require more mature and activated DCs, such as those provided by skin grafts. Indeed, skin-grafting D^d-primed mice have never failed to elicit a memory response. However perplexing, the finding that rB cell populations delayed the memory response *in vivo* is consistent with recent studies demonstrating that $CD8^+$ memory T cells can be tolerized to the same extent as naïve populations [21], and with earlier studies demonstrating that $CD8^+$ T cell clones, representative of memory populations, exhibit split anergy, that is, mediate CTL activity, but fail to proliferate and clonally expand unless supplied with exogenous IL-2 [22, 23] when presented with antigen by quiescent APC.

Although our findings appear to be at odds with other reports in the literature showing that memory T cells can be activated to alloantigen expressed on rB cells [1], such is not the case. As with memory responses to minor-H alloantigen, we too saw preservation of memory CTL responses in mice exposed to alloantigen on rB cells *in vivo* (data not shown). However, skin graft rejection in minor-H primed mice exposed to minor-H disparate rB cells was not tested [1], so it is possible that exposure of minor-H primed mice to minor-H-expressing rB cells, prior to skin grafting, would also have abrogated accelerated rejection.

In conclusion, we have demonstrated that MHC disparate rB cell populations are tolerogenic and that the reason that MHC disparate rB cells derived from normal mice are immunogenic is the presence of small numbers of contaminating dendritic cells. Lastly, we have confirmed the

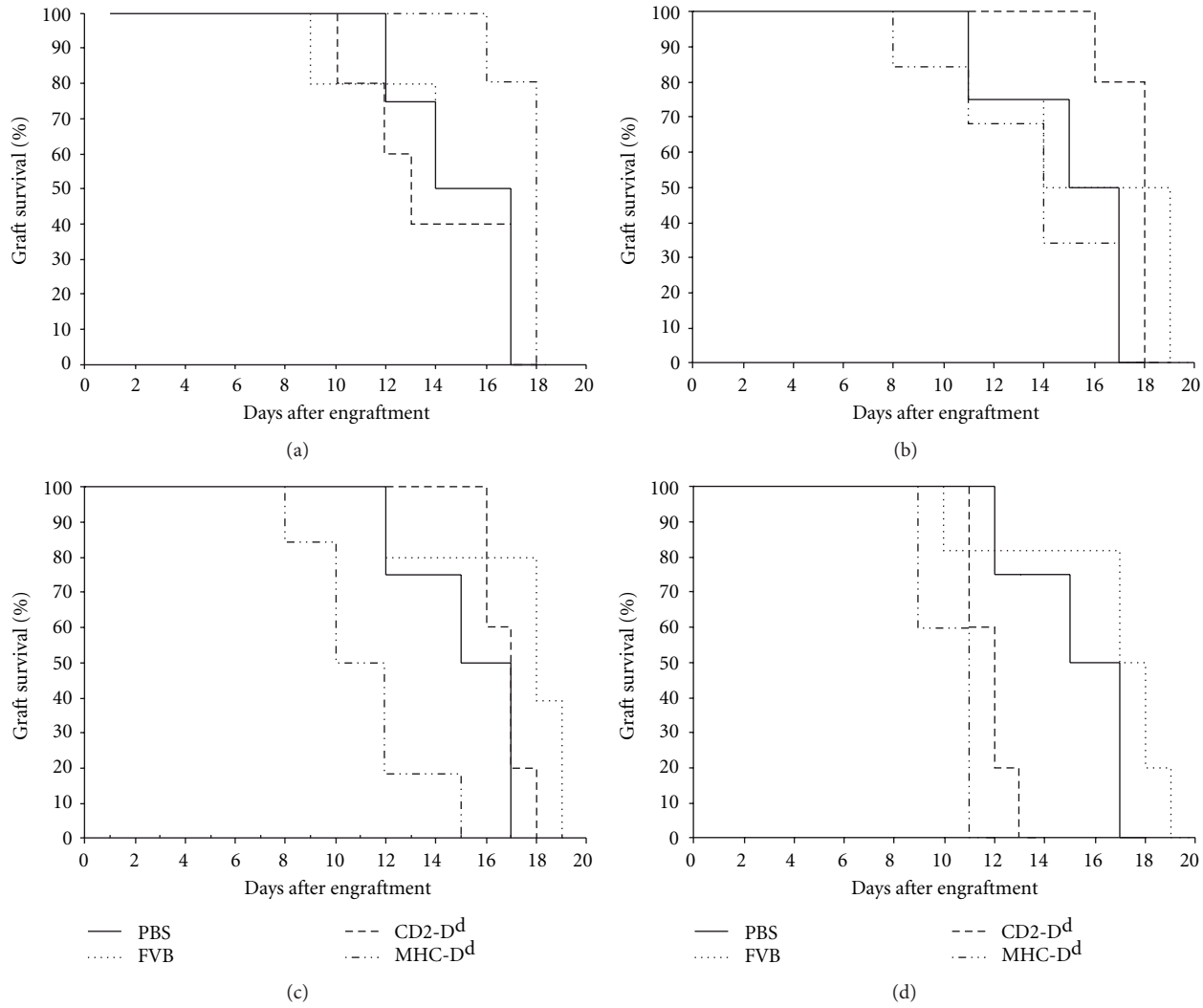

Figure 5: CD2-D^d DC accelerate D^d skin graft rejection. $CD11c^+$ cells from the indicated strains were isolated by MACS magnetic separation and administered i.v. to FVB recipients. Two weeks later, recipient mice were engrafted with MHC-D^d tail skin. Plots A-D represent log doses of DC administered: (a) 10^3 DC, (b) 10^4 DC, (c) 10^5 DC, and (d) 10^6 DC.

susceptibility of memory $CD8^+$ T cell responses to disruption via exposure to tolerogenic lymphocyte populations.

4. Materials and Methods

4.1. Transgenic Mice. CD2-D^d transgenic mice were generated by injecting FVB embryos linking the CD2 promoter with D^d cDNA construct (a kind gift of Dr. Randy Ribaudo) coding sequence in pD^d SELFIX.34 and an appropriate enhancer expression cassette p29Δ2(sal-) [14]. The CD2-D^d mice used were from the 4906 line, which showed the highest level of D^d expression [14]. MHC-D^d transgenic mice were generated by injecting FVB embryos with the genomic D^d gene, including the MHC class I promoter isolated from *pDd1* (a kind gift of Dr. Gilbert Jay) [14]. Mouse studies were performed in accordance with Institutional Animal Care and Use Committee guidelines.

4.2. Mice and Priming. FVB/N mice and thymectomized (ATX) FVB/N mice, an inbred $H\text{-}2^q$ strain, were purchased from Taconic Farms (Germantown, NY, USA). For priming experiments, mice were given a single injection (i.p.) of 2×10^7 spleen cells from MHC-D^d mice at least 3 weeks before treatment.

4.3. Cytofluorometry Analysis. 1 or 2 color analysis was performed by incubating cells with the following PE or FITC-conjugated mAbs: anti-CD11c (PE) (09705B), anti-CD86 (B7.2) (PE) (09275B), anti-CD40 (PE) (09665B), anti-CD4 (L3T4) (PE) (01065A), and anti-D^d (FITC) (06134D) (PharMingen Co, CA Becton Dickinson).

4.4. Preparation of Resting B Cell Populations. Splenocytes from MHC-D^d mice and CD2-D^d mice were depleted of

erythrocytes with ACK lysis buffer (Biosource) and centrifuged over a discontinuous Percoll gradient (Pharmacia) containing 50–60% and 70% layers as described previously [1]. Cells at the 60%–70% interface were collected and depleted of T cells, macrophages, and DC by incubating with biotinylated mAbs (PharMingen) directed against CD3 (01082D), CD4 (09422D), CD8 (01042D), Thy1.1 (22212D), CD11b (Mac-1) (01712D), and CD11c (09702D), then mixed with streptavidin-coated microbeads (Miltenyi Biotec GmbH, Germany) and passed over a MACS separation column (Miltenyi Biotec GmbH). CD2-D^d T cells were prepared by depleting CD2-D^d B cells from the spleen cells of CD2-D^d mice by incubating with biotinylated mAbs directed against B220 and I-A^q, and then adding streptavidin-coated microbeads (Miltenyi Biotec GmbH, Germany) and passage over an MACS separation column (Miltenyi Biotec GmbH).

4.5. Isolation of DC by FACS. $CD11c^+$ cells from MHC-D^d mice were isolated by cell sorting, using positive selection, as described previously [24]. Briefly, low-density spleen cells from MHC-D^d were incubated with PE-CD11c mAb (PharMingen). The suspensions, at 10×10^6 cells/mL of phosphate-buffered saline with 2% FBS and 0.5 mM EDTA, were sorted in an FACStar Plus instrument (Becton Dickinson). Recovered cells contained 91% $CD11c^+$ DC.

4.6. MACS DC Isolation. Briefly, spleens were digested with 1 mL of complete RPMI containing collagenase D (Roche), 331 Mandl U/1 mL and DNase I, and bovine pancreas grade II (Roche) 16.5 ug/mL injected into the spleen and incubated at 37°C for 30 min. Spleens were then minced and passed 5x through a 19 ga needle, passed through a cell strainer (Becton Dickinson), treated with ACK lysing buffer (Biousource), and washed once with PBS containing 0.5% BSA and 2.5 mM EDTA. MACS separations were performed as per manufacturers recommendations using LS columns (Miltenyi Biotec) and anti-CD11c (N418) microbeads (Miltenyi Biotec).

4.7. T Cell Adoptive Transfer. Naive or D^d-primed T cells were prepared by negative selection from spleen cells of naive or primed FVB as described in [25]. Briefly, spleen cell suspensions from naive or primed FVB mice were incubated for 30 minutes on ice with a cocktail of mAbs directed against B220, I-A^q, CD11b, and CD11c mAbs, then were washed, incubated with streptavidin-coated microbeads for 15 min, and depleted by MACS. 3×10^7 of these cells were infused into naive ATX FVB mice that had been irradiated with 500 cGy 1 week previously. For purification of $CD8^+$ T cells by negative selection with MACS, spleen cells from naive or primed FVB mice were depleted of CD4-, CD11b-, CD11c-, B220-, and I-A^q-positive cells using similar techniques, with a resulting $CD8^+$ cell purity of >91.9%. 2×10^7 of these $CD8^+$ cells were infused into naive ATX FVB mice that had been irradiated with 500 cGy 1 week previously.

4.8. Transplant Recipient Treatment. In vivo depletion of $CD4^+$ and/or $CD8^+$ T cells was performed by mAb injection. ATX FVB mice were injected i.p. with GK1.5 (150 μg/mouse) mAb and/or 53.6.7 (200 μg/mouse) mAb on days −6, −5, −1 and +5, with respect to transplantation. Mice were checked for depletion on the day prior to skin grafting [14, 26]. Depletion of $CD4^+$ T cells or $CD8^+$ T cells, or both, from the peripheral blood was >98% in all mice, as assessed by flow cytometry (FACScan, Becton Dickinson). In studies evaluating the role of CD40-CD40L interactions, MR1 (anti-CD40L mAb, 250 μg/mouse) was injected into ATX FVB mice on days −14, −10, −7, −3, 0, 4, and 7 and twice weekly thereafter, with respect to skin transplantation [15]. Anti-D^d sera were prepared by injecting MHC-D^d splenocytes into naive FVB mice i.p. (5×10^6 cells per mouse). The mice received three injections of cells at weekly intervals and 7 days after the last injection they were bled for the purpose of preparing a single pool of antisera. Mice were injected i.v. with 0.2 mL of anti-D^d sera 7, 9, or 12 days after receiving MHC-D^d skin graft.

4.9. Skin Grafting. Tail skin from donor mice was engrafted onto the recipient's flank as described previously [27]. Grafts were scored daily or every other day until they were rejected (>80% loss of graft tissue) .

Abbreviations

MST: Median survival time
ATX: Thymectomized
rB cells: Resting B cells.

Authors' Contributions

K. Tsuji and H. I. McFarland contributed equally to this work.

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