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ORIGINAL ARTICLE

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Minimal conditioning required in a murine model of T cell depletion, thymic irradiation and high-dose bone marrow transplantation for the induction of mixed chimerism and tolerance

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Abstract In a recently developed murine model for the induction of mixed chimerism and tolerance, hosts are treated with T cell depleting monoclonal antibodies (TCD mAbs; days −5, −1 and +7), thymic irradiation (TI) (7 Gy), and a high dose of fully allogeneic bone marrow cells (BMC, 200×10^6). To find the minimum amount of each treatment required for success with this approach, we treated groups with (1) a lower dose of TI (3.5 Gy), (2) fewer BMC (100×10^6), (3) no TI, (4) no TI plus additional TCD mAbs on day +14, or (5) fewer injections of TCD mAbs (day −5 only). Chimerism was followed by flow cytometry (FCM), and tolerance was assessed by skin grafting. Without TI, no long-term chimerism or tolerance could be induced, even when an additional dose of TCD mAbs was administered on day +14. A reduction in the dose of either BMC or TI led to substantially reduced effectiveness, as demonstrated by lower levels of chimerism and poorer donor skin graft survival. However, the dose of TCD mAbs and hence the duration of recipient T cell depletion could be safely reduced and thus the potential toxicity of the conditioning regimen lowered.

Keywords Tolerance · Mixed chimerism · Bone marrow transplantation · T cell depletion

Introduction

The induction of mixed chimerism through hematopoietic stem cell transplantation is a promising approach to the development of a clinically feasible tolerance protocol [22]. A major impediment to the clinical application of this concept is the potential toxicity of the conditioning required for the successful engraftment of allogeneic bone marrow (BM). Our laboratory recently developed a protocol for the induction of mixed chimerism that avoids the need for total body irradiation or cytotoxic drug treatment, which has been required in previous models [14]. In this model, hosts are treated with three doses of TCD mAbs, TI, and a high dose of BMC. While this new protocol constitutes a step towards clinical application of the concept of mixed chimerism, it would be desirable to minimize further the potential toxicity of the conditioning regimen and to reduce the number of hematopoietic cells required. We therefore evaluated whether the dose of TI, BMC, or TCD mAbs could be reduced without loss of effectiveness of the protocol and whether irradiation could be avoided altogether by prolonging the period of TCD.

Materials and methods

Conditioning and BMT

Female C57BL/6 (B6: H-2^b), B10.A (B10.A: H-2^a), and A.SW (H-2^s) mice were maintained in sterilized microisolator cages in which they received autoclaved feed and autoclaved acidified drinking water. Recipient B6 mice received approximately 1.8 mg and 1.4 mg of rat IgG2b anti-mouse CD4 mAb GK1.5 [3] and anti-mouse

CD8 mAb 2.43 [11], respectively, intraperitoneally (i.p.) on the indicated days. Selective thymic irradiation in the indicated dose (3.5 Gy or 7 Gy) was given on day 0 as described [12]. Untreated BMC (approximately 40×10^6 or 20×10^6, respectively) harvested from tibiae and femora of B10.A donors were administered daily on each of days 0 through 4, for a total of five injections (total approximately 200×10^6 or 100×10^6 BMC, respectively). Cyclosporine A (Novartis, East Hanover, N.J., USA), dissolved in olive oil was administered subcutaneously at a dose of 20 mg/kg per day from day −15 to day −3 prior to BMT, as previously described [8]. National Institutes of Health (NIH) guidelines for animal care (NIH publication no. 86–23, revised 1985) were followed in a protocol approved by our institutional animal care and use committee.

Monoclonal antibodies

Nonspecific FcγR binding was blocked by anti-mouse FcγR mAb 2.4G2 [19]. FITC-conjugated mAbs included anti-CD4, anti-CD8, anti-MAC1, anti-B220, and rat anti-mouse IgM, as well as anti-TCR$\alpha\beta$, anti-Vβ5, anti-Vβ11, and anti-Vβ8.1/2 mAbs. Negative control mAb HOPC1-FITC, with no reactivity to mouse cells, was prepared in our laboratory. Biotinylated anti-H–2D^d mAb 34–2-12 [10] and control mAb HOPC1 were developed with phycoerythrin-streptavidin (PEA). Phycoerythrin-conjugated anti-CD4 mAb and nonspecific rat IgG2a (negative control) were also used.

Analysis of multilineage chimerism

Allogeneic reconstitution of various lineages was evaluated by FCM analysis of white blood cells (WBC) at multiple time points. Briefly, forward angle and 90-degree light scatter properties were used to distinguish lymphocytes, monocytes, and granulocytes in peripheral WBC, as described [16]. Two-color FCM was utilized to distinguish donor and host cells of particular lineages, and the percentage of donor cells was calculated as described 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 the total net percentage of donor plus host cells of that lineage.

Analysis of T cell receptor Vβ families

Peripheral blood lymphocytes (PBL) were stained with specific anti-Vβ-FITC mAbs or HOPC1-FITC vs anti-CD4-PE. Flow cytometric analysis was performed on gated CD4$^+$ cells, and staining with control mAb was subtracted to determine the percentage of cells expressing each Vβ in the gated population.

Skin grafting

Skin grafting was performed 9 or 14 weeks after BMT. Full thickness tail skin from B10.A (donor-specific) and fully MHC-mismatched A.SW (H-2^s, third party) mice was grafted onto the lateral thoracic wall, secured with Band-Aids and followed by visual and tactile inspections daily from day 8 onward for 3 weeks, then at least every week thereafter. Grafts were defined as rejected when less than 10% of the graft remained viable.

Statistical analysis

An unpaired Student's *t*-test was used for comparing deletion of donor-reactive CD4$^+$ PBL. *P* values of <0.05 were considered statistically significant.

Results

Chimerism and skin graft survival

As shown previously [14], substantial levels of long-lasting multilineage hematopoietic chimerism were achieved after treatment with three doses of TCD mAbs, 7 Gy TI, and 200×10^6 BMC (Fig. 1A) (for group descriptions, see Table 1). Two of four mice in this group accepted donor skin for more than 100 days, and a third mouse accepted it for 80 days, while all mice promptly rejected third party skin (Table 2). Therefore, donor-specific tolerance was induced in most mice in this group. Treatment of hosts with 3.5 Gy TI instead of 7 Gy resulted in substantially lower mean levels of donor representation among all tested WBC lineages (Fig. 1B). Even though multilineage chimerism was completely undetectable in only one mouse by 27 weeks after BMT, only one of three mice in this treatment group accepted donor skin long-term, while the two others rejected it within 16 days (Table 2). As shown previously, the elimination of TI from this protocol led to low levels of initial chimerism, which rapidly declined, and were soon lost completely (Fig. 1D) [14]. As expected, donor skin grafts were rejected promptly (Table 2). The group of mice receiving 100×10^6 BMC instead of 200×10^6 demonstrated a similar result, with lower levels of chimerism (Fig. 1C), and all three mice rejected donor skin grafts within 3 weeks (Table 2). The administration of an additional dose of anti-CD4 and CD8 depleting mAbs on day 14 instead of TI failed to induce lasting chimerism and tolerance (Fig. 1E, Table 2).

In a separate experiment, we injected a group of animals with only one dose of TCD mAbs (on day −5). These mice developed very similar levels of multilineage chimerism (Fig. 1F) to that seen in the positive control group in the same experiment (not shown), with three of four mice retaining high levels of multilineage chimerism for the duration of follow-up (33 weeks). One donor skin graft was lost due to an apparent infection on day 48, one showed long-term acceptance, and the remaining two rejected on days 21 and 42, respectively (with the mouse showing only transient chimerism rejecting on day 21) (Table 2). In this experiment we also evaluated the effect of host pretreatment with a course of cyclosporine A (CyA) prior to BMT. In a related model for the induction of mixed chimerism, using 3 Gy total body irradiation, 7 Gy TI, one dose of TCD mAbs, and a standard dose of BM, such pretreatment with CyA was shown to obviate the need for TI [8]. However, mice pretreated with CyA, three doses of TCD mAbs, and 200×10^6 BMC but no irradiation ($n=5$) did not show higher levels of chimerism or long-term donor skin graft survival (median survival time 12.5 days) than mice not receiving CyA (data not shown). When 3.5 Gy TI were used in addition to

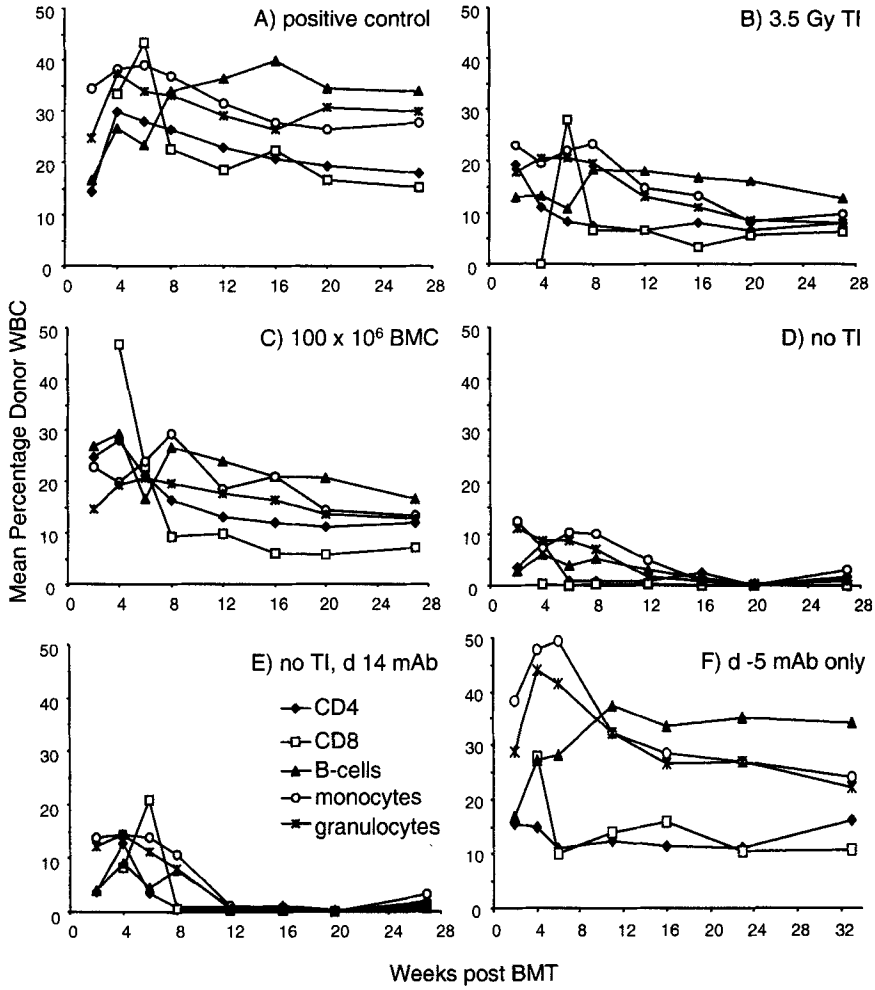

Fig. 1A–F. Mean percentage of donor cells at various times in peripheral blood of mice treated with various modifications of a protocol of T cell depletion, thymic irradiation, and high-dose BMT. (For group descriptions, see also Table 1). **A** Group A ($n=5$) received the previously published successful protocol (14) (positive control). All other groups were treated with modifications of this regimen. Mice received 200×10^6 unseparated fully MHC-mismatched BMC (administered as five doses of 40×10^6 BMC over days 0 to 4), depleting doses of anti-CD4 plus anti-CD8 mAbs on days –5, –1, and +7, and 7 Gy of selective irradiation to the thymic area (day 0). **B** Group B ($n=4$) received the same treatment as group A, except that the dose of TI was reduced to 3.5 Gy. **C** Group C ($n=5$) received the same conditioning as group A, but only half the dose of BMC (100×10^6 BMC, administered as five doses of 20×10^6 BMC over days 0 to 4). **D** Group D ($n=5$) received the same treatment as group A, except without TI. **E** Group E ($n=4$) received no TI and an additional dose of TCD mAbs on day +14. **F** Group F ($n=4$) was treated with TCD mAbs on day –5, 7 Gy TI, and 200×10^6 BMC

CyA pretreatment and the standard TCD mAbs and BMC doses ($n=5$), only one mouse developed long-term chimerism, and donor skin grafts were rejected with a median survival time of 11 days (data not shown).

Deletion of donor-reactive T cells

The donor strain B10.A expresses I-E, which is required to present superantigens derived from the mammary tumor virus (Mtv)-8 and –9 endogenous retroviruses encoded in the B6/B10 background genome. Developing thymocytes whose T cell receptors (TCR) contain Vβ11 or Vβ5.1/2, which bind to these superantigens, are deleted in I-E-positive B10.A mice [1, 5, 18] but not in B6 mice, because they do not express I-E [2, 18]. Central deletion has been shown to be the main mechanism for the maintenance of tolerance through mixed chimerism [14, 16]. We therefore determined the frequency of Vβ11$^+$ and Vβ5.1/2$^+$ CD4$^+$ cells in PBL 18 weeks after

Table 1. Treatment groups. *TCD* T cell depleting, *BMC* bone marrow cells, *mAb* monoclonal antibody, *CyA* Cyclosporin A

	Thymic irradiation	BMC dose	TCD mAbs	CyA pretreatment
Group A	7 Gy	200×10^6	d–5, d–1, d+7	No
Group B	3.5 Gy[a]	200×10^6	d–5, d–1, d+7	No
Group C	7 Gy	100×10^{6a}	d–5, d–1, d+7	No
Group D	0 Gy[a]	200×10^6	d–5, d–1, d+7	No
Group E	0 Gy[a]	200×10^6	d–5, d–1, d+7, d+14[a]	No
Group F	7 Gy	200×10^6	d–5[a]	No
Group CyA-1	0 Gy[a]	200×10^6	d–5, d–1, d+7	Yes[a]
Group CyA-2	3.5 Gy[a]	200×10^6	d–5, d–1, d+7	Yes[a]

[a]Indicates the parameter(s) differing from group A (positive control)

Table 2. Skin graft results. Skin graft survival (in days) in mice treated with various modifications of a protocol of high-dose BMT, T-cell depletion, and thymic irradiation. Third-party and donor skin was grafted 9 or 14 weeks after BMT, respectively. For group descriptions, see legend to Fig. 1. Note: one mouse each in groups A and E died before skin grafting. *TF* technical failure

	Graft survival			Graft survival	
	Third party	Donor		Third party	Donor
Group A (positive control)			Group B (3.5 Gy TI)		
#1	11	>100	#1	13	>100
#2	11	80	#2	16	16
#3	11	23	#3	11	TF
#4	11	>100	#4	11	11
Group C (100×10^6 BMC)			Group D (no TI)		
#1	11	TF	#1	14	13
#2	11	11	#2	TF	TF
#3	11	TF	#3	14	14
#4	11	21	#4	14	14
#5	11	12	#5	12	15
Group E (no TI, d14 mAb)			Group F (d5 mAb only)		
#1	14	14	#1	9	>48
#2	12	18	#2	12	21
#3	TF	TF	#3	10	42
			#4	8	>100

BMT. Mice of the positive control group receiving the full protocol demonstrated a profound reduction in the percentages of $V\beta11^+$ and $V\beta5^+$, but not $V\beta8^+$ $CD4^+$ PBL (Fig. 2), suggesting effective deletion of donor-reactive thymocytes. The groups treated with either less TI or fewer BMC both showed results similar to those of the positive controls, though there was a nonsignificant trend towards less reduction of $V\beta11^+$ and $V\beta5^+$ $CD4^+$ PBL. The two groups receiving no TI, in contrast, demonstrated substantially higher levels of $V\beta5^+$ and $V\beta11^+$ $CD4^+$ PBL (Fig. 2), consistent with the absence of long-term chimerism.

Discussion

In the studies described herein, we explored possibilities for reducing the severity of the host conditioning and reducing donor marrow cell numbers in a model using TCD mAbs, TI, and high-dose BMT for the induction of mixed chimerism and tolerance. In syngeneic strain combinations, it was shown that engraftment of BMC could be achieved in unconditioned hosts by the administration of very high doses of BMC [13, 15]. The introduction of mixed chimerism protocols into clinical practice has so far largely been prevented by the potential toxicity of the required host conditioning, especially any form of recipient irradiation. Here we demonstrate that while a combination of TCD mAbs, TI, and high doses of fully allogeneic BM allowed the induction of long-lasting macrochimerism, neither a prolonged period of profound T cell depletion nor pretreatment with CyA could overcome the need for local irradiation to the thymic area. A 50% reduction in the dose of TI led to a significant loss of effectiveness of the protocol which could not be compensated for by CyA pretreatment. In a related model that includes a low dose of total body irradiation, CyA pretreatment permitted allogeneic BM

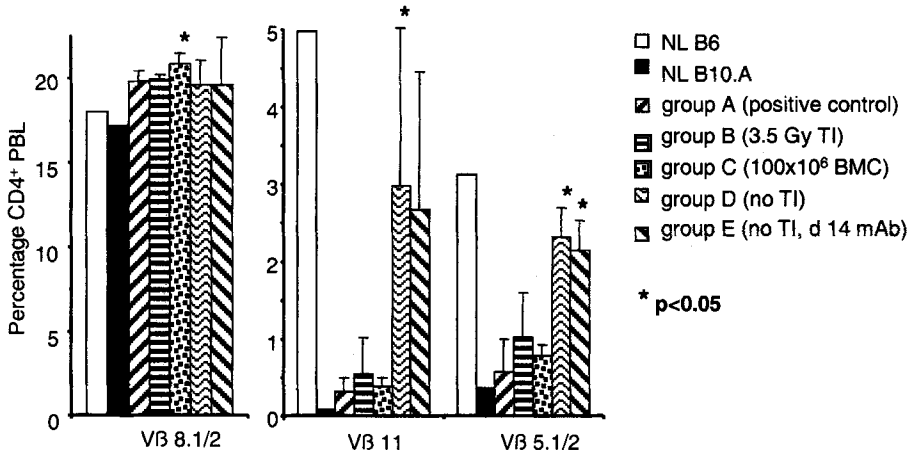

Fig. 2. Deletion of donor-reactive $CD4^+$ PBL 18 weeks post-BMT. Results are shown as mean. Error bars indicate standard deviation. *P* value was calculated for comparison with group A (positive control). For group descriptions, see Fig. 1 and Table 1. Group A ($n=4$), group B ($n=4$), group C ($n=5$), group D ($n=5$), group E ($n=3$). *NL* normal

engraftment without TI by creating thymic "space" and/or overcoming intrathymic alloresistance [8]. In the absence of total body irradiation, as in the current studies, however, CyA pretreatment was not sufficient to improve outcome significantly. Thymic irradiation seems to have a dual effect in this model. Firstly, it facilitates BM engraftment considerably ([15] and Wekerle, Ito, and Sykes, unpublished data), which can possibly be explained by the fact that significant areas of the sternum, ribs, and vertebrae are included in the irradiation field. Secondly, TI helps to overcome intrathymic alloresistance [9] and thus reduces overall alloreactivity.

Regarding the dose and duration of TCD, a single dose of TCD mAbs prior to BMT proved sufficient to allow induction of lasting chimerism and tolerance. Additional doses of TCD mAbs did not improve outcome and could not replace TI, suggesting that the BM engraftment-promoting effect of TI is crucial in this model. Several experimental models for the induction of macrochimerism and tolerance, including studies in nonhuman primates [7] have so far relied in their conditioning protocols on a combination of T cell cytotoxic antibodies and irradiation or myelotoxic drugs [22]. The mAbs available for murine studies, however, accomplish a more complete depletion of T cells, especially in lymphoid organs other than the blood, than can be achieved by high doses of ATG in a related nonhuman primate renal transplant model [7]. It thus seems unlikely that currently clinically available T cell-depleting reagents are capable of overcoming alloreactivity to a degree that would allow BM engraftment without any form of irradiation.

The use of costimulatory blocking mAbs has recently permitted the development of radiation-free protocols for the induction of mixed chimerism and tolerance ([4, 23] and Ito and Sykes, unpublished data). When the same dose of allogeneic BMC as given in the studies described herein 200×10^6 per mouse) is administered together with single injections of anti-CD154 and CTLA4Ig, mixed chimerism and tolerance can be successfully achieved [23]. So why is profound T cell depletion unable to accomplish similar results? Two main hurdles have to be overcome to induce mixed chimerism: nonimmunological engraftment barriers and immunological alloresistance. Theoretically, costimulation blockers could promote BM engraftment by making "space" in the BM compartment. This seems unlikely, however, since costimulatory blockade had no effect on levels of syngeneic marrow engraftment (Kurtz and Sykes, unpublished data).

Since an influence on nonimmunological barriers to engraftment appears unlikely, we speculate that costimulatory blocking reagents may be more effective than T cell depletion in overcoming intrathymic alloresistance. The use of cytotoxic anti-CD4 and anti-CD8 mAbs in doses as described in the studies in this manuscript affords a very profound elimination of T cells from blood and peripheral lymphoid organs but not from the thymus [12, 17]. Surviving donor-reactive thymocytes or T cells must be tolerized or eliminated, which apparently can be achieved through TI [9], anti-CD154, or CTLA4Ig [21]. Mature donor-reactive T cells have been shown to be deleted peripherally after BMT with costimulatory blockade [20, 23], and this could be one of the mechanisms for tolerizing donor-reactive T cells once they leave the thymus. In addition, an active regulatory mechanism, either intrathymically or peripherally, could contribute to tolerizing persisting donor-reactive T cells after BMT with costimulatory blockade [6]. In clinical practice, equivalent doses of hematopoietic stem cells as those given in our high-dose BMT protocol could not readily be obtained from a cadaveric donor. While the use of peripheral mobilized hematopoietic stem cells can increase the number of stem cells obtained from living related donors, these numbers do not approach the numbers given on a per kg basis in our animal model. It would thus be desirable to find the minimum amount of hematopoietic stem cells necessary for the successful induction of mixed chimerism. In the present studies, a reduction of the dose of BM by 50% led to insufficient levels of chimerism to achieve lasting tolerance in most mice. Thus, application of this approach may await the development of effective in vitro stem cell expansion techniques.

In summary, only the dose of TCD mAbs could be reduced without loss of efficacy in a model using TCD mAbs, TI, and high-dose BMT as host conditioning for the induction of mixed chimerism and tolerance. Thus, it seems that TCD needs to be combined with [21] or replaced by [4, 23] costimulatory blocking reagents in order to achieve radiation-free protocols for the induction of mixed chimerism and tolerance.

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