Cell-Cell Interaction in Graft Rejection Responses: Induction of Anti-Allo-Class I H-2 Tolerance Is Prevented by Immune Responses against Allo-Class II H-2 Antigens Coexpressed on Tolerogen

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Summary

The intravenous sensitization of C57BL/6 (B6) mice with class I H-2-disparate B6-C-H-2^{bm1} (bm1) spleen cells results in almost complete abrogation of anti-bm1 $CD8^+$ helper (proliferative and interleukin 2–producing) T cell (Th) activities. Although an appreciable portion of $CD8^+$ cytotoxic T lymphocyte (CTL) precursors themselves remained after this regimen, such a residual CTL activity was eliminated after the engrafting of bm1 grafts, and these grafts exhibited prolonged survival. In contrast, the intravenous sensitization with (bm1 × B6-C-H-2^{bm12} [bm12])F_1 cells instead of bm1 cells failed to induce the prolongation of bm1 graft survival as well as bm12 and (bm1 × bm12)F_1 graft survival. In the (bm1 × bm12)F_1-presensitized B6 mice before as well as after the engrafting of bm1 grafts, anti-bm1 CTL responses that were comparable to or slightly stronger than those observed in unpresensitized mice were induced in the absence of anti-bm1 Th activities. bm1 graft survival was also prolonged by intravenous presensitization with a mixture of bm1 and bm12 cells but not with a mixture of bm1 and (bm1 × bm12)F_1 cells. The capacity of $CD4^+$ T cells to reject bm12 grafts was eliminated by intravenous presensitization with antigen-presenting cell (APC)-depleted bm12 spleen cells. However, intravenous presensitization with APC-depleted (bm1 × bm12)F_1 cells failed to induce the prolongation of bm1 graft survival under conditions in which appreciably prolonged bm12 graft survival was induced. More surprisingly, bm1 graft survival was not prolonged even when the (bm1 × bm12)F_1 cell presensitization was performed in $CD4^+$ T cell–depleted B6 mice. This contrasted with the fact that conventional class I–disparate grafts capable of activating self Ia-restricted $CD4^+$ as well as allo-class I–reactive $CD8^+$ Th exhibited prolonged survival in $CD4^+$ T cell–depleted, class I–disparate cell-presensitized mice. These results indicate that: (*a*) intravenous presensitization with class I– and II–disparate cells fails to reduce anti-allo-class I rejection responses that would otherwise be eliminated using only class I–disparate cells; (*b*) such failure is generated according to the coexpression of both classes of alloantigens on a single cell as tolerogen; and (*c*) allo-class II antigens coexpressed on tolerogen function to activate $CD4^+$ as well as non-$CD4^+$ Th leading to the generation of anti–class I effector T cell responses.

An immune response to antigen is, in general, modulated by various factors/conditions, including the route via which antigen is introduced into an individual (1). The presentation of alloantigens via the intravenous route regulates positively or negatively the induction of anti-allo-immune responses. This has been reported for the effect of donor-specific transfusion (DST)[1] in humans (1–8) as well as for the effect of donor-specific intravenous presensitization (DSP) with allogeneic cells in animal studies (9–17).

The fact that there are different outcomes after DST/DSP depends on the disparity of the donor-host combination as well as the blood cell component used for the intravenous presensitization (11–14, 16, 17). For example, we have demonstrated that the DSP of C57BL/6 (B6) mice with class I H-2-disparate allogeneic cells (B6-C-H-2^{bm1} [bm1]) resulted in the abrogation of $CD8^+$ Th activity against the bm1 alloantigens (15) and produced prolonged survival of bm1 skin graft (16). In contrast, DSP with class II H-2-disparate

[1] *Abbreviations used in this paper:* DSP, donor-specific intravenous presensitization; DST, donor-specific transfusion; FMF, flow microfluorometric; SN, supernatants.

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allogeneic spleen cells (B6-C-H-2^{bm12} [bm12]) failed to induce tolerance of $CD4^+$ Th reactive to bm12 alloantigens (17). These findings indicated that similar protocols of immunomodulation produce distinct effects on the capacity of $CD8^+$ and $CD4^+$ Th subsets. The combination of class I and class II H-2 disparities induces heterogenous subsets of Th (18–20). Therefore, the above observations raise the question of whether anti–class I and class II immune pathways are independently affected by DSP with class I– and class II–disparate cells, or whether the effect of DSP on one pathway is influenced by an alternative pathway. Investigating such a DSP effect could contribute to a better understanding of DSP/DST-induced complicated immunomodulation in the combinations with a complex disparity.

The present study was undertaken to investigate how anti–class I H-2 (bm1) rejection responses are modulated when class I– and class II–disparate (bm1 × bm12)F_1 cells, which also have the potential to induce anti–class II immune responses, are used for DSP instead of only class I–disparate (bm1) cells. The results demonstrate that DSP of B6 mice with bm1 cells resulted in the reduction of anti-bm1 rejection responses along with the elimination of anti-bm1 $CD8^+$ Th activity. In contrast, the DSP protocol using (bm1 × bm12)F_1 cells instead of bm1 cells failed to reduce anti-bm1 rejection potential under conditions in which anti-bm1 Th activity was eliminated similarly to the effect of DSP with bm1 cells. The rejection of bm1 grafts in B6 mice after DSP with (bm1 × bm12)F_1 cells was associated with a potent induction of anti-bm1 CTL responses. This contrasted with the suppressed CTL generation in B6 mice receiving DSP with bm1 cells, suggesting that potent CTL responses are induced by cellular interactions between anti-bm1 CTL precursors and anti-bm12 Th. It was also found that the generation of anti-bm1 CTL and graft rejection responses were not induced when a mixture of bm1 and bm12 cells was used for DSP instead of (bm1 × bm12)F_1 cells. These results indicate that an immune response against a given alloantigen that would otherwise be tolerized by DSP with cells expressing only the alloantigen is activated through the introduction of DSP-resistant immune responses to other alloantigens coexpressed on DSP cells. The results are discussed in the context of the nature of the DSP-resistant immune responses.

Materials and Methods

Mice. C57BL/6 (B6) and BALB/c mice were purchased from Shizuoka Experimental Animal Laboratory. B6-C-H-2^{bm1} (bm1), B6-C-H-2^{bm12} (bm12), and B10.QBR mice were originally provided by Dr. D. H. Sachs (National Cancer Institute, Bethesda, MD). These three strains of mice and hybrid (bm1 × bm12)F_1 mice were bred in our laboratory. These mice were used for experiments at 7–9 wk of age.

Intravenous Presensitization with Allogeneic Cells. Mice were injected with 10^7 allogeneic bm1 or (bm1 × bm12)F_1 spleen cells via the intravenous route.

Media Used for In Vitro Cell Culturing. Culture medium used for the MLR was RPMI 1640 supplemented with 10% horse serum, 5×10^{-5} M 2-ME, sodium pyruvate, glutamine, nonessential amino acids, and 5 mM Hepes buffer. Complete medium for the generation of CTL was prepared by replacing 10% horse serum with 10% FCS. RPMI 1640 supplemented with 5% FCS plus 5×10^{-5} M 2-ME was used for the production of IL-2 and IL-2 assays.

Monoclonal Antibodies. Anti-CD4 (GK1.5) (21) and anti-CD8 (3.155) (American Type Culture Collection, Rockville, MD) mAbs were obtained from culture supernatants of hybridomas producing the relevant antibody. GK1.5 mAb was also obtained in the form of ascitic fluid. A gamma globulin fraction of the ascitic fluid was obtained by precipitation at 50% saturation with ammonium sulfate and used for in vivo administration.

Mixed Lymphocyte Reaction. The responding lymph node cells, obtained from a pool of three to five animals per group, were resuspended in complete medium and cultured at a concentration of 2×10^5 cells per culture well together with 4×10^5 irradiated (2,000 rad) stimulating spleen cells in a total volume of 0.2 ml (15–17). Each MLR was performed in triplicate in flat-bottomed microculture plates (Corning 25860; Corning Glass Works, Corning, NY) and maintained in humidified atmosphere at 5% CO_2 at 37°C. The cultures were harvested onto glass fiber filters with an automatic cell harvester at 96 h after an 8-h pulse with 20 KBq/well of [^{3}H]TdR. Radioactivity was determined by liquid scintillation spectrometry. Results were calculated from the uptake of [^{3}H]TdR and expressed as the mean uptake in cpm ± SE of triplicate cultures.

Generation of Cytotoxic T Lymphocytes. CTL induction was performed as previously described (22). Briefly, 4 or 5×10^6 spleen cells were cultured in vitro with 10^6 irradiated (2,000 rad) syngeneic or allogeneic spleen cells in 24-well culture plates (Corning 25820; Corning Glass Works) in a volume of 2 ml at 37°C for various periods. Effector cells generated were assayed on ^{51}Cr-labeled target cells (spleen cells cultured for 48 h with Con A, 5 μg/ml). Percent specific lysis was calculated as described (22). SE in each group were <10% and these were excluded from figures for simplicity.

Production of IL-2 and Assay System for IL-2 Activity. Spleen cells (5×10^6) were cultured with 3×10^6 syngeneic or allogeneic stimulator spleen cells (2,000 rad) in 24-well culture plates (Corning 25820; Corning Glass Works) in a volume of 1 ml (15–17). After incubation at 37°C in a humidified incubator (5% CO_2) for 48 h, culture supernatants (SN) were harvested by centrifugation and stored at −20°C until use.

SN were assayed for IL-2 activity according to their ability to support the proliferation of the IL-2-dependent T cell line, CTLL-2 (15–17). CTLL-2 (10^4/well) were cultured with SN in a volume of 0.2 ml in 96-well flat-bottomed microplates (Corning 25860; Corning Glass Works) for 24 h at 37°C. Proliferation was assessed by the uptake of [^{3}H]TdR during 4-h pulsing with 20 KBq [^{3}H]TdR/well.

Skin Grafting of B6 Mice. Mice were engrafted on the flank with tail skin grafts according to an adaptation of the method of Billingham and Medawar (23). Bandages were removed on day 7 and the grafts were scored daily until rejection (defined as loss of >80% of the grafted tissue).

Depletion of Mac-1$^+$ Cells (APC) from Spleen Cells. A single cell suspension of spleen cells was depleted of Mac-1^+ cells (APC) by two round passages over Sephadex G-10 columns as described by Ly and Mishell (24). Depletion of Mac-1^+ cells (<1%) was confirmed by flow microfluorometric (FMF) studies using anti-Mac-1 mAb (M1/70).

Depletion of CD4$^+$ T Cells. The procedure was the same as described (25). Semipurified anti-CD4 antibody (GK1.5) was administered in vivo at the dose of 100 μg/d three times/wk. Ad-

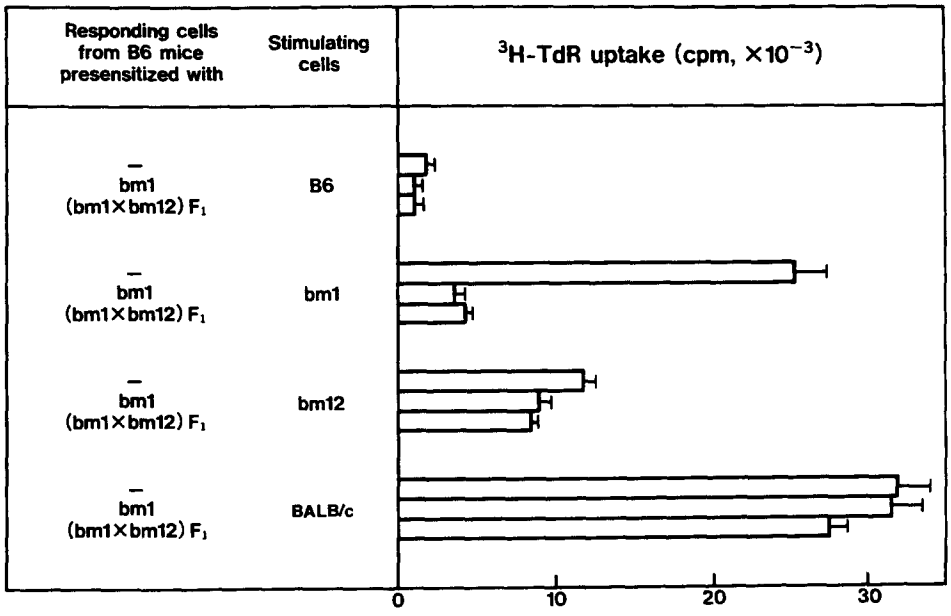

Figure 1. Suppression of anti-bm1 MLR capacity by DSP with (bm1 × bm12)F_1 cells. B6 mice were injected intravenously with 10^7 bm1 or (bm1 × bm12)F_1 cells (DSP). 1 wk later, lymph node cells (2×10^5) from these presensitized and unpresensitized B6 mice were stimulated in vitro with irradiated spleen cells (4×10^5) from indicated strains of mice for 96 h in 96-well microculture plates.

ministration of the antibody for >2 wk resulted in almost complete elimination of $CD4^+$ T cells as revealed by FMF study (25, 26).

Results

Suppression of Anti-bm1 Th Activities by DSP with bm1 or (bm1 × bm12)F_1 Spleen Cells. B6 mice were presensitized intravenously with 10^7 bm1 or (bm1 × bm12)F_1 spleen cells (DSP). 1 wk after DSP, lymph node cells from these mice were tested for their ability to proliferate upon in vitro stimulation with bm1, bm12, or BALB/c spleen cells (MLR). As shown in Fig. 1, lymph node cells from either bm1- or (bm1 × bm12)F_1-presensitized B6 mice exhibited markedly reduced anti-bm1 MLR. Spleen cells from the same presensitized mice as used in Fig. 1 were also tested for their capacity

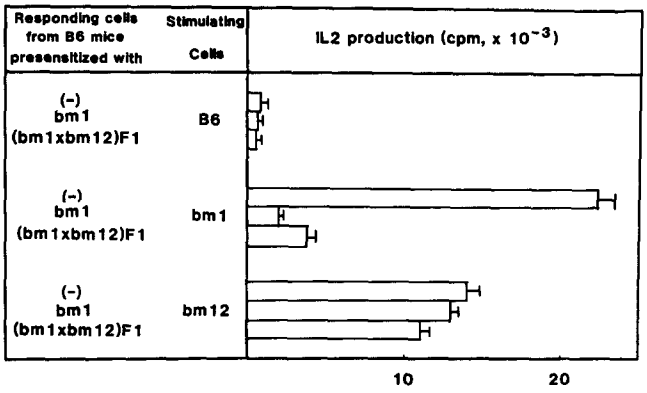

Figure 2. Suppression of anti-bm1 IL-2 production by DSP with (bm1 × bm12)F_1 cells. Spleen cells (5×10^6) from the same mice as used in Fig. 1 were cultured with irradiated stimulator spleen cells (3×10^6) in 24-well plates. SNs were assayed for IL-2 activity using the IL-2-dependent cell line, CTLL-2.

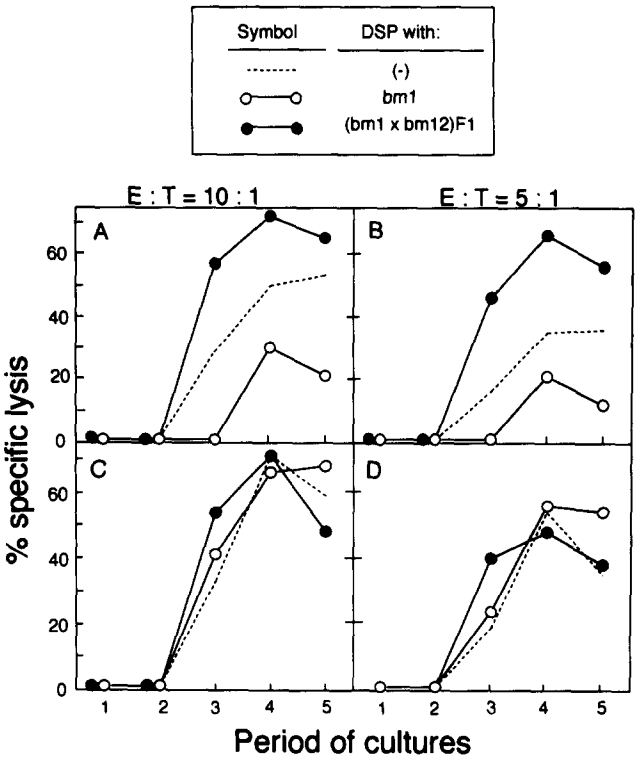

Figure 3. Enhanced anti-bm1 CTL potential in mice presensitized with (bm1 × bm12)F_1 cells. Spleen cells (4×10^6) from unpresensitized mice or mice presensitized with bm1 or (bm1 × bm12)F_1 cells were stimulated in vitro with irradiated bm1 (*A* and *B*) or BALB/c (control) (*C* and *D*) spleen cells (10^6) in 24-well culture plates for 1–5 d. Responding cells recovered various days after culturing (effector cells) were tested for their anti-bm1 (*A* and *B*) or anti-BALB/c (*C* and *D*) cytotoxic activity on bm1 or BALB/c blast target cells at E/T ratios of 10:1 and 5:1.

to produce IL-2 upon stimulation with bm1 or bm12 alloantigens (Fig. 2). The results demonstrate that DSP with cells expressing bm1 antigens resulted in a reduction of the capacity to produce IL-2 in response to the stimulation with bm1 alloantigens, and also indicate that DSP with bm1 or (bm1 × bm12)F_1 cells eliminates anti-bm1 Th activities as measured by MLR and IL-2 production.

Enhanced Induction of Anti-bm1 CTL Responses by Spleen Cells from B6 Mice after DSP with (bm1 × bm12)F_1 Cells. To determine the effect of DSP with bm1 or (bm1 × bm12)F_1 cells on the ability to generate anti-bm1 CTL responses, spleen cells from bm1- or (bm1 × bm12)F_1-presensitized mice were stimulated in vitro with bm1 or BALB/c (control) cells. Effector cells generated various periods (1–5 d) later were assayed on bm1 or BALB/c blast target cells (Fig. 3). The results demonstrate that spleen cells from bm1-presensitized B6 mice produce anti-bm1 CTL responses, but the CTL activity is appreciably weaker compared to that obtained with normal B6 spleen cells, which confirms our previous results (16). In contrast, DSP with (bm1 × bm12)F_1 cells resulted in a considerably enhanced generation of anti-bm1 CTL responses when responding cells from these presensitized mice were stimulated in vitro with bm1 cells. The results of Fig. 3 also illustrate that such modulation was specific for anti-bm1 CTL responses, since comparable magnitudes of anti-BALB/c CTL responses were generated by spleen cells from either normal, bm1-presensitized, or (bm1 × bm12)F_1-presensitized B6 mice. Thus, DSP of B6 mice with (bm1 × bm12)F_1 cells results in the enhancement of anti-bm1 CTL potential along with almost complete elimination of anti-bm1 Th activity.

Differential Effects of DSP with bm1 vs. (bm1 × bm12)F_1 Cells on bm1 Graft Survival. We next investigated the effects of DSP with (bm1 × bm12)F_1 cells on the generation of anti-bm1 graft rejection responses. The finding shown in Fig. 4 (a part of results) confirms that DSP of B6 mice with bm1 cells induces prolongation of bm1 graft survival. In contrast, when DSP was performed using (bm1 × bm12)F_1 cells, the prolongation of bm1 graft survival was not observed, but rather a slightly accelerated graft rejection was seen.

We examined anti-bm1 T cell (Th and CTL) responsiveness in B6 mice engrafted with bm1 grafts after DSP with (bm1 × bm12)F_1 cells (Table 1 and Fig. 5). The results of Table 1 demonstrate that lymph node cells from B6 mice engrafted with bm1 grafts (7-d graft bearing) after DSP with either bm1 or (bm1 × bm12)F_1 cells exhibit the suppression of anti-bm1 Th (MLR and IL-2 production) activities on one hand. On the other, spleen cells from bm1- or (bm1 × bm12)F_1-presensitized mice 7 d after bm1-engrafting generated markedly reduced or potent (slightly higher) anti-bm1 CTL responses when compared with those obtained by unpresensitized, bm1 skin-bearing control mice (Fig. 5). These results indicate that T cell (Th and CTL) responsiveness as observed in B6 mice receiving only DSP with (bm1 × bm12)F_1 cells continues after engrafting of bm1 skin grafts. Taken together with the results of Fig. 4, Table 1, and Fig. 5, the failure of (bm1 × bm12)F_1-presensitized mice to induce the prolongation of bm1 graft survival is associated with the potent anti-bm1 CTL response that is induced under conditions in which the suppression of anti-bm1 Th activities is continued.

Suppression of Prolongation of bm1 Graft Survival Depends on DSP with Cells Coexpressing bm1 and bm12 Antigens. The preceding observations suggested that a concomitant anti-bm12 immune response generated through DSP with (bm1 × bm12)F_1 cells functioned to prevent the prolongation of bm1 graft survival that would otherwise be induced by DSP

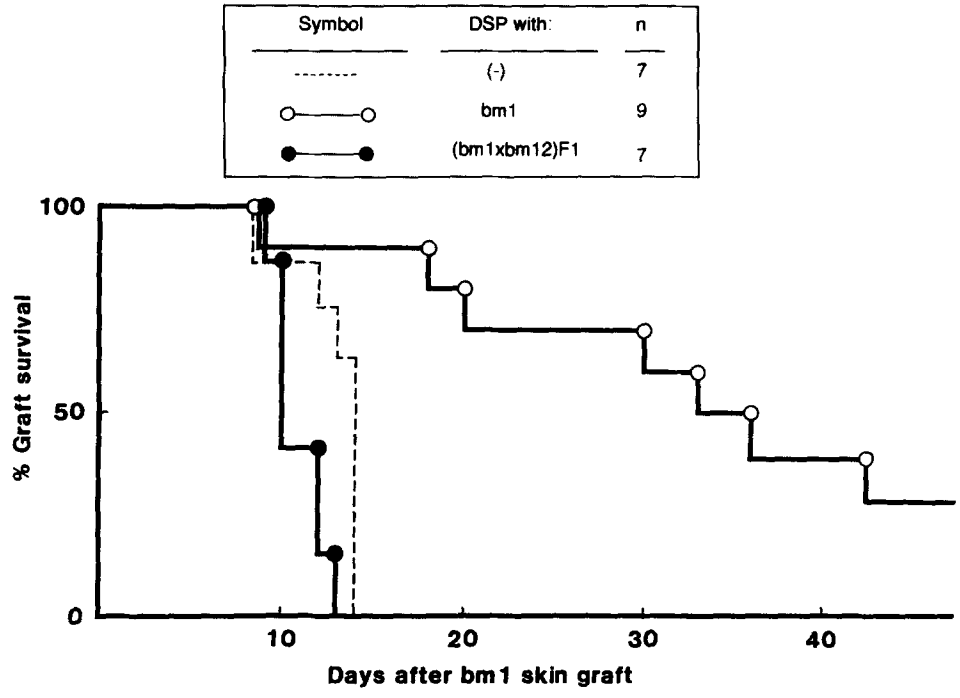

Figure 4. Survival of bm1 skin graft on B6 mice presensitized with bm1 or (bm1 × bm12)F_1 cells. B6 mice were presensitized intravenously with 10^7 bm1 or (bm1 × bm12)F_1 cells. 1 wk later, these presensitized and unpresensitized mice were engrafted with bm1 grafts.

Table 1. *Suppression of Anti-bm1 Th Activities in B6 Mice Engrafted with bm1 Grafts after DSP with bm1 or (bm1 × bm12)F₁ Cells*

Assay*	Responding cells from B6 mice: DSP with:	Responding cells from B6 mice: engrafted with:	[³H]TdR uptake (cpm, ×10⁻³) after stimulation with: B6	bm1	BALB/c
MLR	-	bm1	2.6 ± 1.0	29.6 ± 1.0	26.5 ± 0.7
	bm1	bm1	3.7 ± 0.1	4.4 ± 0.4	24.1 ± 0.4
	(bm1 × bm12)F₁	bm1	3.5 ± 0.1	8.5 ± 0.1	19.3 ± 0.7
IL-2 production	-	bm1	0.3 ± 0.0	12.3 ± 0.7	15.9 ± 0.6
	bm1	bm1	0.2 ± 0.0	2.2 ± 0.1	17.7 ± 0.4
	(bm1 × bm12)F₁	bm1	0.4 ± 0.1	1.2 ± 0.5	13.2 ± 1.0

* Lymph node cells (for MLR assay) or spleen cells (for IL-2 production) from B6 mice engrafted with bm1 grafts after DSP with indicated cells were stimulated with bm1 or BALB/c (control) spleen cells. In IL-2 production, culture SNs were assayed for IL-2 activity using the CTLL-2 cell line.

with bm1 cells. We further investigated the conditions required for the generation of such an anti-bm12 immune response. B6 mice received DSP with either (bm1 × bm12)F₁ cells, a mixture of bm1 and bm12 cells, or a mixture of bm1 and (bm1 × bm12)F₁ cells 1 wk before the engrafting of bm1 grafts. Fig. 6 again shows a slightly accelerated rejection of bm1 grafts by DSP with (bm1 × bm12)F₁ cells. The results also demonstrate that: (*a*) bm1 grafts engrafted in B6 mice presensitized with a mixture of bm1 and bm12 cells exhibited a comparable degree of prolonged survival to that observed in B6 mice presensitized with bm1 cells (Fig. 4), and (*b*) DSP with a mixture of bm1 and (bm1 × bm12)F₁ cells failed to induce the prolongation of bm1 graft survival. These findings indicate that the concomitant generation of anti-bm12 immune responses along with DSP leads to the prevention of prolongation of bm1 graft survival, but this is the case only when anti-bm12 responses are induced by bm12 antigens coexpressed with bm1 antigens on a single DSP cell. Moreover, these observations also exclude the possibility that the failure of (bm1 × bm12)F₁ cells to produce the anti-bm1 DSP effect is ascribed to the expression of bm1 antigens on F₁ cells either in an incomplete way or in a manner different from that on bm1 cells.

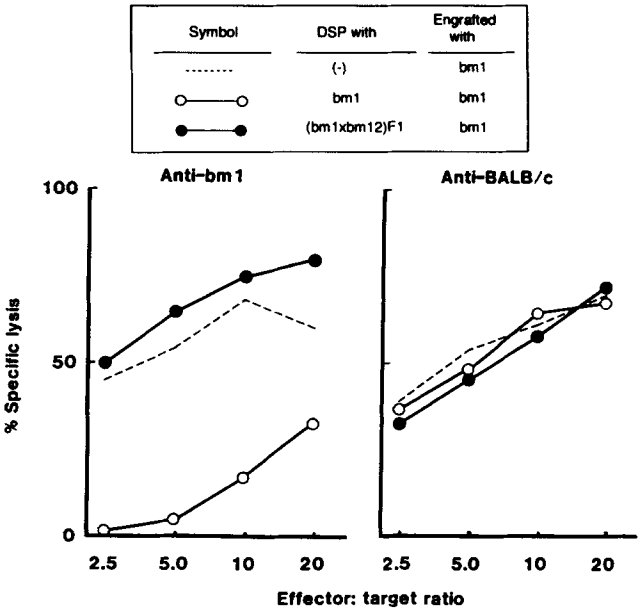

Figure 5. The generation of potent anti-bm1 CTL responses by B6 mice engrafted with bm1 after DSP with (bm1 × bm12)F₁ but not with bm1 cells. Portions of the same spleen cells as used in the IL-2 production in Table 1 were sensitized in vitro with bm1 or BALB/c stimulator cells. Effector cells generated 5 d after culturing were assayed on bm1 or BALB/c blast target cells.

Anti-bm12 Non-CD4⁺ T Cell-mediated Inhibition of Anti-bm1 DSP Effect. bm12 antigens have been known to induce the activation of Th (proliferative/IL-2 producing) and T cells responsible for anti-bm12 graft rejection that are exclusively of CD4⁺ phenotype (17). An earlier study from our laboratory has also demonstrated that DSP of B6 mice with APC-depleted bm12 spleen cells (essentially bm12 antigen-expressing B cells) but not with whole spleen cells results in the elimination of anti-bm12 CD4⁺ Th and graft rejection-mediating T cells (17). Therefore, we finally investigated whether the anti-bm1 DSP effect is inducible even by DSP with (bm1 × bm12)F₁ cells under conditions in which anti-bm12 CD4⁺ T cell activity is not induced. B6 mice received DSP with various types of cells: (*a*) bm1 cells, (*b*) Sephadex G-10-passed bm12 (G-10-bm12), (*c*) mixture of bm1 and G-10-bm12 cells, (*d*) (bm1 × bm12)F₁ cells, and (*e*) G-10-(bm1 × bm12)F₁ cells. 1 wk later, unpresensitized and these presensitized B6 mice were engrafted with either bm1, bm12, or (bm1 × bm12)F₁ grafts. The results are summarized in Fig. 7. The results confirm that the anti-bm1 DSP effect is not inducible with cells coexpressing bm1 and bm12 antigens ([bm1 × bm12]F₁ cells) (*A*) and that DSP with G-10-bm12 cells induces marked prolongation of bm12 skin graft survival (*B*). Moreover, Fig. 7 provides the following important information. First, DSP with a mixture of bm1 and G-10-bm12 cells induces the prolongation of (bm1 × bm12)F₁

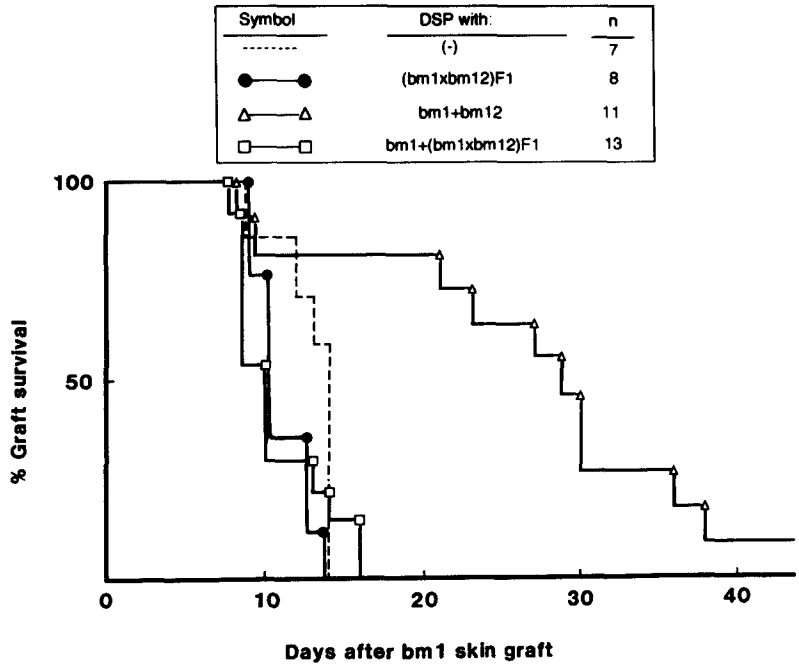

Figure 6. Coexpression of bm1 and bm12 antigens on DSP cells is required for blocking anti-bm1 DSP effect. B6 mice were presensitized with either (bm1 × bm12)F_1 cells (10^7), a mixture of bm1 (10^7) and bm12 cells (10^7), or a mixture of bm1 (10^7) and (bm1 × bm12)F_1 cells (10^7) 1 wk before engrafting bm1 grafts.

graft survival (*C*). These observations exclude the possibility of the existence of F_1-unique antigens (27) that would be expressed only on F_1 cells but not on bm1 or bm12 cells. This suggests that the blockage of the anti-bm1 DSP effect at the time of DSP with F_1 cells is produced through cellular interactions between anti-bm1 effector cells and assisting (Th) cells directed against bm12 but not against putative F_1-unique antigens.

Second, DSP with G-10-(bm1 × bm12)F_1 cells was seen to induce appreciable levels of prolongation of bm12 graft survival. Even under these conditions, however, portions of the same DSP cells failed to induce any significant prolongation of bm1 graft survival (Fig. 7 *A*). In fact, the bm1 graft rejection in this DSP group was slightly accelerated, similar to that observed in the (bm1 × bm12)F_1-DSP group when compared with an unpresensitized control group. These results indicate that the anti-bm1 DSP effect is not produced by DSP with G-10-F_1 cells capable of reducing anti-bm12 $CD4^+$ T cell reactivity. This implies the existence of an alternate anti-bm12 immune response pathway that influences anti-bm1 responses upon DSP with F_1 or G-10-F_1 cells.

Additional experiments were performed to confirm that anti-bm12 responses involved in the blockage of the anti-bm1 DSP effect do not necessarily belong to the $CD4^+$ Th subset. B6 mice were depleted of $CD4^+$ T cells by injecting semipurified anti-CD4 (GK1.5) antibody at the dose of 100 μg/d three times per week as previously described (25, 26). This procedure was capable of almost completely eliminating $CD4^+$ T cells when continued for 2 wk (six times) (data not shown here but detailed in references 25 and 26). B6 mice were given the anti-CD4 antibody according to the above protocol. 1 wk after the initiation of anti-CD4 injections, B6 mice were presensitized with untreated or G-10-passed (bm1 × bm12)F_1 cells, and 1 wk later, engrafted with bm1 skin grafts. The administration of the anti-CD4 antibody was continued until the grafts were rejected. The results are shown in Fig. 8 *A*. The anti-CD4 administration failed to produce the anti-bm1 DSP effect after DSP with either (bm1 × bm12)F_1 or G-10-(bm1 × bm12)F_1 cells. This contrasted with the beneficial effect of the anti-CD4 treatment on the prolongation of graft survival in two other control combinations (Fig. 8, *B* and *C*): the B6-bm12 combination in which allo-class II H-2-restricted $CD4^+$ Th (graft rejection–initiating T cells) are functioning and the B6-B10.Q.R combination with class I H-2 disparity in which self class II H-2-restricted $CD4^+$ Th and allo-class I H-2-restricted $CD8^+$ Th are functioning along with $CD8^+$ CTL precursors. In the former combination, the administration of anti-CD4 antibody resulted in striking prolongation of bm12 graft survival (Fig. 8 *B*). In the latter, prolongation of B10.QBR grafts was not induced by either DSP with B10.QBR cells or anti-CD4 treatment. However, the combination of these treatments produced an appreciable level of prolongation of B10.QBR graft survival, which is consistent with the previous finding (26). In this model, DSP with B10.QBR together with the anti-CD4 treatment eliminated the respective allo-class I–restricted $CD8^+$ Th and self class II–restricted $CD4^+$ Th (see reference 26). The observations in Fig. 8, *B* and *C* illustrate that the anti-CD4 treatment is capable of eliminating various $CD4^+$ Th subsets (allo-class II restricted and self class II restricted). Taken collectively, the results of Fig. 8 indicate that an alternate anti-bm12 immune response that is not mediated by the $CD4^+$ T cell subset is activated upon DSP with (bm1 × bm12)F_1 cells and that this response

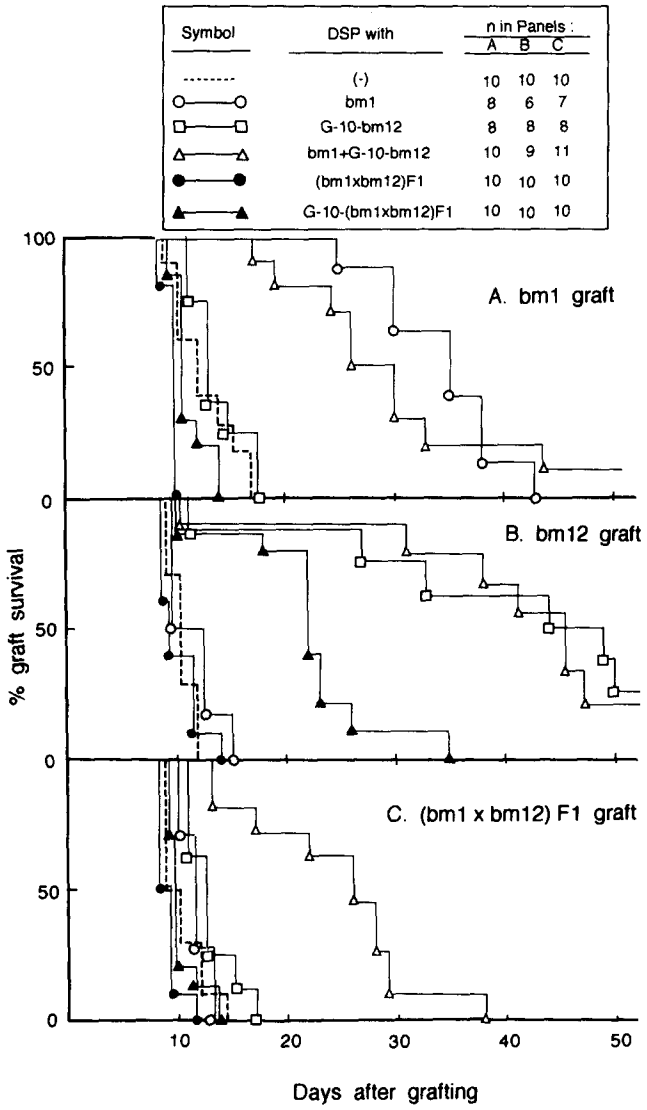

Figure 7. Differential effects of DSP with untreated or G-10-passed spleen cells on survival of bm1, bm12, or (bm1 × bm12)F_1 grafts. B6 mice were presensitized with indicated DSP cells 1 wk before engrafting bm1, bm12, or (bm1 × bm12)F_1 grafts.

functions to induce the generation of anti-bm1 CTL and graft rejection responses under conditions in which the classical anti-bm12 responses ($CD4^+$ T cells) are eliminated.

Discussion

Immune responses to alloantigens are initiated by the recognition of alloantigens by helper-type T cells and by their subsequent production of various lymphokines capable of activating effector T cells. Therefore, elimination and/or functional suppression of Th cells lead to an essential immunomodulation of anti-allo-immune responses. For example, DSP of B6 mice with class I MHC-disparate bm1 cells results in the elimination of anti-bm1 allo-class I–restricted $CD8^+$ Th activity (15, 16). Because this represents the abrogation of the total Th activity functioning in the B6-bm1 combination, DSP in this combination leads to the reduction of in vivo rejection potential and prolongation of graft survival (16). In contrast to allo-class I–restricted $CD8^+$ Th, some of other Th subsets appear to be resistant to immunomodulation by DSP (26). In considering that various subsets of Th would function in the donor-host combinations with complex disparities such as combinations with conventional class I and/or class II disparities, the question has been raised of how the immunomodulation of DSP-sensitive responses to alloantigen A is influenced by DSP-resistant responses to alloantigen B upon DSP with cells expressing both A and B alloantigens.

The present study was undertaken to determine how cellular interactions between different subsets of Th and effector T cells influence the production of DSP effects on each pathway of the immune responses. It was demonstrated that: (*a*) DSP of B6 mice with class I–disparate bm1 cells or class I– and class II–disparate (bm1 × bm12)F_1 cells resulted in almost complete abrogation of anti-bm1 Th activity; (*b*) DSP with bm1 induced prolongation of bm1 graft survival whereas DSP with (bm1 × bm12)F_1 cells failed to prolong bm1 graft survival; (*c*) the failure of DSP with (bm1 × bm12)F_1 cells to produce anti-bm1 DSP effect was associated with the enhanced induction of anti-bm1 CTL responses before and after bm1 skin grafting that was not observed by DSP with bm1 cells; (*d*) DSP with a mixture of bm1 and bm12 cells produced an anti-bm1 DSP effect, indicating that the coexpression of both alloantigens on a single DSP cell is necessary to block the generation of the anti-bm1 DSP effect; and (*e*) surprisingly, even when the $CD4^+$ T cell subset including the major anti-bm12 Th is eliminated, DSP with (bm1 × bm12)F_1 cells failed to prolong bm1 graft survival.

The present results would permit discussion from two mutually related aspects. First, it was demonstrated that there was a fundamental biological difference between DSP using cells with only class I- vs. class I + class II disparities in the suppression of anti–class I MHC responses. It may be worth considering that hosts that have been presensitized intravenously with class I– and class II–disparate ([bm1 × bm12]F_1) cells possess enhanced potential to reject only class I–disparate grafts as well as class I– and II–disparate grafts. This enhanced potential would be reflected on potent anti–class I (bm1) CTL responses generated when spleen cells from the (bm1 × bm12)F_1-presensitized host are stimulated in vitro with bm1 cells. Thus, such enhanced anti–class I immune potential would be elaborated in the course of DSP with cells coexpressing class I and class II antigens on a single DSP cell. The production of beneficial DSP/DST effects would therefore be more difficult in donor-host combinations with class I and class II disparities than in class I–disparate, class II–compatible combinations. This is based on the fact that in addition to the operation of anti–class II responses themselves, these responses function to interfere with the reduction of anti–class I responses. However, even if it is supposed that a graft from the (class I + class II)-disparate donor consists mostly of class I-positive class II-negative cells, it is impor-

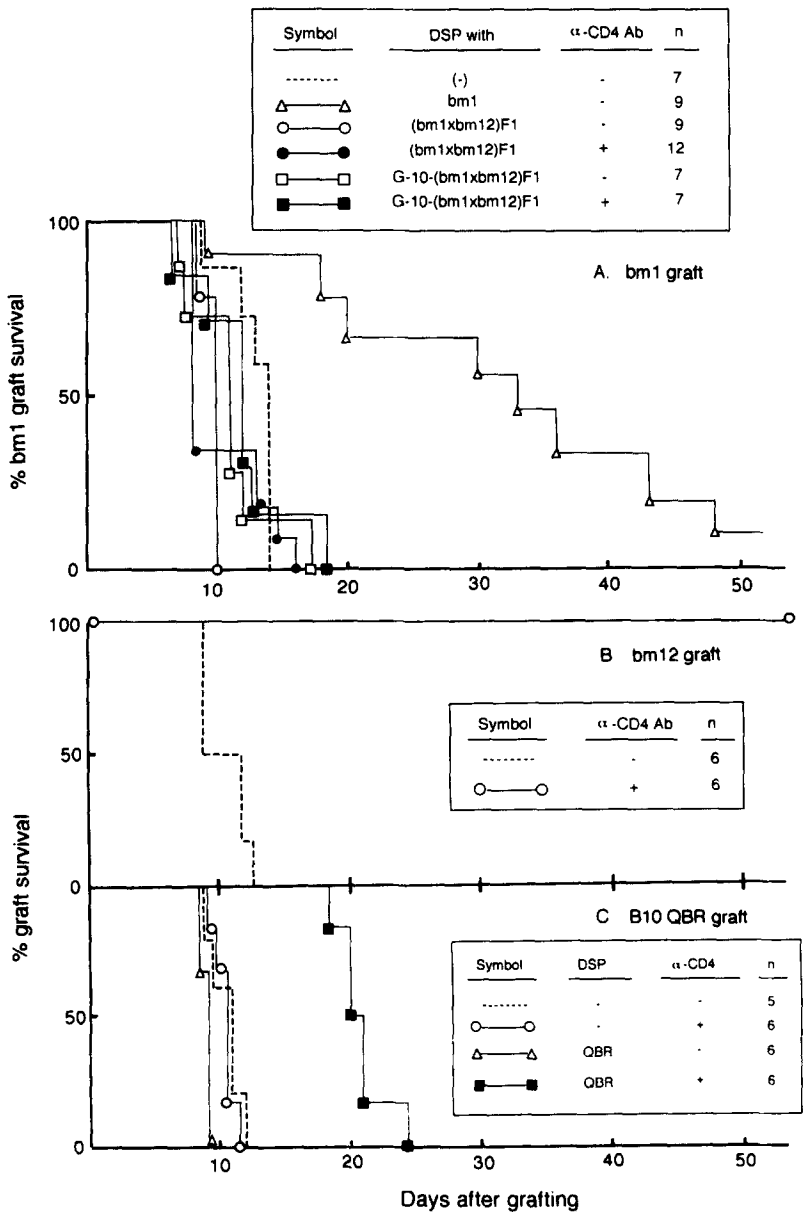

Figure 8. Failure of anti-CD4 administration to produce anti-bm1 DSP effect after DSP with (bm1 × bm12)F_1 cells. B6 mice were given anti-CD4 antibody (100 μg/time, three times/wk). 1 wk after initiation of anti-CD4 administration, these mice were not presensitized or were presensitized with indicated strains of spleen cells. 1 wk later, these mice were engrafted with grafts from indicated strains of mice.

tant to note that anti-class I immune reactivity is enhanced at the timing of DSP/DST.

The second aspect is concerned with the nature of anti-bm12 responses that would function to enhance anti-bm1 CTL responses along with DSP with (bm1 × bm12)F_1 cells. It is reasonable to assume that the suppression of the anti-bm1 DSP effect upon DSP with (bm1 × bm12)F_1 cells is produced by the introduction of some element(s) of anti-bm12 reactivity. In general, anti-bm12 T cell responses have been found to be mediated exclusively by the $CD4^+$ T cell subset (17, 20). The function of this T cell subset includes the capacities to induce MLR/IL-2 production in vitro (17, 20) and to initiate graft rejection in vivo (20). DSP of B6 mice with bm12 spleen cells failed to eliminate these capacities. However, they were almost completely eliminated by DSP with APC-depleted bm12 spleen cells (bm12 B cells) (20, 28). While taking these findings into consideration, the present study sought to determine whether APC-depleted (bm1 × bm12)F_1 spleen cells function to produce the anti-bm1 DSP effect. The results demonstrated that DSP with APC-depleted (bm1 × bm12)F_1 (G-10-[bm1 × bm12]F_1) cells induced appreciable levels of the anti-bm12 DSP effect, as observed by the prolongation of bm12 skin graft survival. Even under these conditions, however, the same regimen failed to produce an anti-bm1 DSP effect. More surprisingly, the anti-bm1 DSP effect was not produced in $CD4^+$ T cell-depleted B6 recipient mice when (bm1 × bm12)F_1 cells were used as DSP donor cells. It is unlikely that an immune response to F_1-unique antigen (27) is involved in the blockage of the anti-bm1 DSP effect, because such a response is not detected in B6 mice depleted of anti-bm1 and -bm12 responses (Fig. 7 *C*). Alternatively, the above observations may also suggest

that anti-bm12 responses mediated by non-CD4$^+$ T cells are involved in the enhanced generation of anti-bm1 CTL as well as graft rejection potential upon DSP with (bm1 × bm12)F_1 cells.

Three major Th subsets have been described as functioning in the recognition of allo-class I and -class II MHC antigens (19). These include: (*a*) allo-class I–restricted CD8$^+$ Th (e.g., anti-bm1 Th); (*b*) allo-class II–restricted CD4$^+$ Th (e.g., anti-bm12 Th); and (*c*) self class II–restricted allo-class I–reactive CD4$^+$ Th (although a bm1 mutant class I antigen does not induce this type of Th, they are commonly induced in combinations with conventional class I-disparities; references 26 and 29). Thus, two types of Th (a and b) are considered to function in the B6-(bm1 × bm12)F_1 combination (16, 17). We have found difficulty in understanding the present results in terms of the above-described idea. Several groups of investigators have described the existence of CD4$^+$ and CD8$^+$ CTL reactive to allo-class II MHC antigens (30–32). Moreover, Shinohara et al. (33) found that some of these CTL recognize class II alloantigens in the context of self MHC antigens (33), indicating that there exist CTL subsets that differ from one another in their phenotype and mode of alloantigen recognition. Likewise, the possibility can not be ruled out that additional distinct subsets of Th exist to be activated with class I or class II alloantigens. In this context, some of our recent findings regarding the establishment of phenotypically and functionally heterogenous bm12-specific Th clones appear relevant (Iwata, S., A. Kosugi, and H. Fujiwara, manuscript in preparation). An anti-bm12 Th line was established from long-term cultures of B6 spleen cells with irradiated bm12 spleen cells. By subsequent cell cloning, 30 bm12-specific cloned lines were obtained. Approximately 90% of these T cell clones were CD4$^+$ with a highly proliferative capacity, whereas the rest were mildly proliferative CD8$^+$. These observations demonstrated the existence of anti-bm12 Th clones that have not been described previously. The postulation that such anti-bm12 CD8$^+$ Th functioned to assist anti-bm1 CTL responses upon DSP with (bm1 × bm12)F_1 cells could account for the enhanced generation of anti-bm1 CTL potential under the conditions of complete CD4$^+$ T cell depletion.

Even though anti-bm12 responses consist of distinct subsets of Th, their characteristics will also have to be taken into consideration in comparison with those of other Th subsets. Earlier studies from our laboratory focused on the heterogeneity of CD8$^+$ and CD4$^+$ Th functioning in conventional class I–disparate combinations according to their DSP susceptibility as well as their capacity to exhibit MLR (26, 29). The results demonstrated that: (*a*) allo-class I–restricted CD8$^+$ Th had potent proliferative (MLR) capacity and were highly susceptible to DSP (29), as has been observed for anti-bm1 CD8$^+$ Th (16); (*b*) self class II–restricted allo-class I–reactive CD4$^+$ Th consisted of highly proliferative and less proliferative (undetectable in MLR) subsets (26) that were susceptible and resistant to DSP, respectively (29); and (*c*) the former CD4$^+$ Th subset could function to initiate graft rejection without activating CTL precursors (CTL-independent graft rejection pathway), whereas the latter functioned exclusively as Th assisting CTL responses (CTL-Th) (29). As an analogy to this, it may be possible to speculate on the heterogeneity of allo-class II–reactive Th subsets from their phenotypes as well as their DSP susceptibility and MLR capacity. Allo-class II–restricted CD4$^+$ Th with highly proliferative capacity (classical anti-bm12 CD4$^+$ Th) have been found to be susceptible to DSP with B cells, but not with APC, expressing bm12 alloantigens (28). It appears that these Th cells function to initiate the CTL-independent graft rejection pathway in a manner similar to self class II–restricted allo-class I–reactive CD4$^+$ Th with potent MLR capacity. Regarding the above allo-class II–reactive CD8$^+$ anti-bm12 Th subset, it remains unclear how numerous these Th cells are among bulk spleen cells. However, if we assume that they function as CTL-Th and are resistant to DSP just as self class II–restricted allo-class I–reactive CD4$^+$ Th with less proliferative capacity, the present results may be more easily understood. In this postulation, this subset of Th (CTL-Th) in B6 recipient mice might be visualized as being resistant to DSP with (bm1 × bm12)F_1 cells and functioning to assist the activation of anti-bm1 CD8$^+$ CTL precursors. Such cell-cell interaction would occur even in CD4$^+$ T cell–depleted recipient mice. It is also conceivable that in the B6-bm12 combination, this subset of Th would neither be detectable in MLR nor function in anti-bm12 graft rejection due to a lack of sufficient anti-bm12 CTL precursors.

In conclusion, the present study illustrates that compared to the disparity at only class I MHC, the disparity at class I and class II MHC induces a more potent activation of anti–class I CTL and graft rejection responses through the introduction of anti–class II Th capable of assisting anti–class I responses. Further, the suppression of the anti–class I response that would otherwise be induced by DSP in only class I disparity is blocked upon DSP in the class I + class II–disparate combination through more complicated cellular interactions. Finally, the anti–class II response involved in such cellular interactions is regarded as a new type of Th that has as yet not been observed in the class II–disparate combination. Thus, by demonstrating that there exists a new pathway of anti–class II responses leading to the activation of anti–class I responses through cellular interactions, and that such a pathway induces complicated modulation of anti–class I graft rejection potential at the timing of DSP, the present study could provide important insights into transplantation immunology.

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