

EVIDENCE FOR INVOLVEMENT OF DUAL-FUNCTION  
T CELLS IN REJECTION OF MHC CLASS I  
DISPARATE SKIN GRAFTS

Assessment of MHC Class I Alloantigens as In Vivo  
Helper Determinants

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A major issue in transplantation immunology is the nature of the cellular interactions and responses involved in in vivo responses to tissue allografts. In previous work, we demonstrated that skin allograft rejection is initiated and mediated only by T cell populations that contained both lymphokine-secreting T helper ( $T_h$ )<sup>1</sup> cells and cytolytic T effector ( $T_{eff}$ ) cells reactive against the alloantigens of the graft (1). We further showed that skin allograft rejection could result from interactions between  $T_h$  cells and  $T_{eff}$  cells of different Lyt phenotypes and of different antigen specificities (1). The present study specifically addresses the cellular pathways and interactions involved in the rejection of MHC class I disparate grafts. Though rejection of MHC class I disparate skin grafts can result from an interaction between  $L3T4^+$  class II-specific  $T_h$  cells and  $Lyt-2^+$  class I-specific  $T_{eff}$  cells, rejection of MHC class I disparate grafts can also be mediated by isolated  $Lyt-2^+$  T cell populations (1-3). However, uncertain in this latter mechanism is whether the  $T_h$  and  $T_{eff}$  cellular functions, contained within the isolated  $Lyt-2^+$  T cell population and requisite to reject class I disparate skin allografts, are mediated by functionally distinct populations of  $T_h$  and  $T_{eff}$  cells or by single populations of dual-function  $T_h/T_{eff}$  cells (4-6). To address these two possibilities, we tested whether class I-specific  $T_h$  cells provided help in vivo for the activation of physically distinct populations of  $T_{eff}$  cells capable of rejecting skin allografts.

In the present study, we show that class I-reactive T cells reject skin allografts for which they are specific, but, unlike class II-reactive T cells, fail to generate help for the rejection of third-party skin allografts. Nevertheless, Class I-allospecific  $T_h$  cells do recognize and respond to class I allodeterminants expressed on skin grafts, but the responding T cells consume nearly all of the lymphokine produced. These findings are most consistent with the participation in anti-class I rejection responses of dual-function  $T_h/T_{eff}$  cells that consume the helper lymphokines that they secrete.

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<sup>1</sup> *Abbreviations used in this paper:* MST, median survival time;  $T_{eff}$ , T effector;  $T_h$ , T helper.

### Materials and Methods

*Mice.* B10 *nu/nu* female mice were obtained from the Small Animal Section, National Institutes of Health, Bethesda, MD. C57BL/6 (B6) and C57BL/10 (B10) mice were obtained from The Jackson Laboratory, Bar Harbor, ME. B6.T1a<sup>a</sup> breeder mice were a generous gift of Dr. L. Flaherty, Albany, NY (7). B10.QBR, B6.C-H-2<sup>bm1</sup> (bm1) (8), B6.C-H-2<sup>bm12</sup> (bm12) (9), B6.C-H-2<sup>bm6</sup> (bm6) (10), and F<sub>1</sub> mice were bred in our own animal facility.

*mAbs.* Anti-L3T4 mAb was either a culture supernatant of the hybridoma cell line GK1.5 (11) or was an ascites of the hybridoma cell line RL-172/4 (12). Anti-I-A<sup>b</sup> mAb was a culture supernatant of the hybridoma cell line M5/114 (13). Rat anti-murine IL-2-R mAb was a mouse ascites of the hybridoma cell line 7D4 (14).

*Cell Fractionation.* Depletion of L3T4<sup>+</sup> T cells was accomplished by incubating spleen cells at a density of 10<sup>7</sup> cells/ml with anti-L3T4 (1:2 dilution of GK1.5 or a 1:100 dilution of RL-172/4) mAb for 30 min at 4°C. Cells were then pelleted, resuspended, and incubated with complement for 50 min at 37°C. The cells treated with GK1.5 were resuspended at 10<sup>7</sup>/ml in low toxicity rabbit complement (Cedar Lane, Ontario, Canada) diluted 1:10. The cells treated with RL-172/4 were resuspended at 10<sup>8</sup>/ml in Guinea pig complement (Gibco Laboratories, Chagrin Falls, OH) diluted 1:3. Treated cells were washed three times before injection into experimental animals or placement in culture as indicated.

*Skin Grafting of Normal B6 Mice and B10 nu/nu Mice.* Mice were engrafted on the left thorax with two tailskin grafts, separated by a host skin bridge, according to an adaptation of the method of Billingham and Medawar (15). Bandages were removed on day 7, and the grafts were scored daily until rejection or the end point of the experiment.

*Adoptive Transfer.* Female B10 nude mice were engrafted as above. On day 1, the mice were injected intravenously with 5 × 10<sup>7</sup> spleen cells from unprimed female B6 mice that had been untreated or treated with anti-L3T4 antibody and complement. Bandages were removed on day 7, and the grafts were scored daily until rejection or the end point of the experiment. This adoptive transfer model has been described in detail elsewhere (1).

*Culture Conditions.* Responder and stimulator populations were obtained either from primed or unprimed mice as indicated. Spleen stimulator cells were irradiated with 2,000 rad. Responder populations were depleted of adherent accessory cells by passage over Sephadex G-10 columns (16). Responder spleen cells (4 × 10<sup>5</sup>) and stimulator spleen cells (4 × 10<sup>5</sup>) were cultured together for 4 d in 0.2 ml of culture medium at 37°C in 7.5% CO<sub>2</sub>, as previously described (16), unless otherwise indicated. Where indicated, 0.01% ascites of anti-IL-2-R mAb, 7D4, was added to each culture to inhibit the consumption of IL-2 during the response period (17). On day 4 of culture, 0.1 ml of supernatant was obtained from each well and assayed by its ability to maintain the growth of an IL-2-dependent cell line, HT-2 (18). Control experiments showed that the IL-2 content of the supernatants from these response cultures reached a plateau on day 4. After 24 h, cultures of HT-2 cells were pulsed with 1 μCi of [<sup>3</sup>H]thymidine, incubated for an additional 12–18 h, then harvested. Background counts of HT-2 cells pulsed with medium alone varied between 200 and 1,200 cpm; maximum counts of HT-2 cells pulsed with an excess of IL-2 varied between 60,000 and 80,000 cpm. The concentration of IL-2 present in the culture supernatants was always limiting, in that the HT-2 response they stimulated always declined in a log-linear fashion as the culture supernatants were diluted. Data are expressed as the arithmetic mean counts per minute of triplicate or quadruplicate cultures. Data as presented are the actual counts obtained and have not been corrected for HT-2 background counts. SE were generally <5% of the mean and so have not been included in the tables.

### Results

*Failure of MHC Class I Alloantigens to Function In Vivo as Helper Determinants for Third-party Rejection Responses.* We have previously demonstrated that most B6 mice most recruit T<sub>h</sub> cells with additional recognition specificities in order to initiate rejection responses against either K<sup>bm6</sup> or Qa-1<sup>a</sup> disparate tailskin allografts (1, 3). To assess the ability of MHC class I allodeterminants to function as in vivo helper de-

terminants for the initiation of rejection responses against third-party  $\text{bm6}$  or  $\text{Qa-1}^a$  skin allografts, we engrafted normal B6 mice with two skin grafts: an "indicator" graft on the lower part of the flank expressing only the target antigen; and an "inducer" graft on the upper part of the flank expressing both the target antigen and putative helper determinants. We then assessed the ability of the inducer graft to promote rejection of the indicator graft. In Table I, we examined the ability of inducer grafts expressing various helper determinants to initiate rejection of  $\text{bm6}$  indicator grafts. It can be seen that inducer grafts expressing only target  $\text{K}^{\text{bm6}}$  antigens failed to induce rejection of  $\text{bm6}$  indicator grafts (group 1). However,  $(\text{bm6} \times \text{bm12})\text{F}_1$  inducer grafts expressing both target  $\text{K}^{\text{bm6}}$  antigens and  $\text{I}^{\text{bm12}}$  determinants, but not  $(\text{B10} \times \text{bm12})\text{F}_1$  inducer grafts expressing  $\text{I}^{\text{bm12}}$  determinants alone, initiated efficient rejection of indicator  $\text{bm6}$  grafts (groups 2 and 3). Thus, consistent with our previous observations (1), MHC class II  $\text{I}^{\text{bm12}}$  allodeterminants were able to elicit T cell help necessary for the local activation of anti- $\text{K}^{\text{bm6}}$  effector cells mediating the rejection of  $\text{bm6}$  indicator skin allografts. More importantly, we also tested the ability of MHC class I ( $\text{K}^{\text{bm1}}$ ) allodeterminants to function as in vivo "helper" determinants for this in vivo rejection response. We did so by using  $(\text{bm6} \times \text{bm1})\text{F}_1$  skin as the inducer graft so that it would express both  $\text{K}^{\text{bm6}}$  target antigens as well as putative  $\text{K}^{\text{bm1}}$  helper determinants. Expression of  $\text{K}^{\text{bm1}}$  allodeterminants by the inducer graft provided a potent rejection stimulus as demonstrated by the rapid rejection of the inducer graft itself (group 4). However, expression by the inducer graft of  $\text{K}^{\text{bm1}}$  class I allodeterminants failed to initiate a significant rejection response against the  $\text{bm6}$  indicator graft (group 4). Similar results were obtained with  $(\text{bm6} \times \text{bm10})$  inducer grafts expressing  $\text{K}^{\text{bm10}}$  class I allodeterminants (data not shown). Thus, unlike MHC class II alloantigens, MHC class I alloantigens failed to generate help for the initiation of third-party anti- $\text{K}^{\text{bm6}}$  rejection responses.

In Table II, we reassessed the ability of  $\text{K}^{\text{bm1}}$  class I allodeterminants to function as in vivo helper determinants using  $\text{Qa-1}^a$  disparate skin allografts from  $\text{B6.T1a}^a$  mice as the indicator grafts. Inducer grafts expressing only  $\text{Qa-1}^a$  target antigens failed to induce rejection of  $\text{Qa-1}^a$  indicator grafts (group 1), whereas inducer grafts expressing both  $\text{Qa-1}^a$  target antigens and  $\text{I}^{\text{bm12}}$  helper determinants did induce rejection of  $\text{Qa-1}^a$  indicator grafts (group 2). However, inducer grafts expressing both  $\text{Qa-1}^a$  target antigens and  $\text{K}^{\text{bm1}}$  class I allodeterminants, despite being highly immunogenic as indicated by rejection of the inducer grafts themselves, failed to initiate rejection of the  $\text{Qa-1}^a$  indicator grafts (group 3). Thus, MHC class I  $\text{K}^{\text{bm1}}$  allodeterminants also failed to generate help for the initiation of anti- $\text{Qa-1}^a$  rejection responses.

*Presence of MHC Class I Allodeterminants Does not Suppress the Rejection of Indicator Skin Grafts Bearing Third-party  $\text{K}^{\text{bm6}}$  Determinants.* We think that  $\text{K}^{\text{bm1}}$  allodeterminants expressed by skin grafts fail to induce the rejection of skin grafts expressing third-party  $\text{K}^{\text{bm6}}$  or  $\text{Qa-1}^a$  determinants because they fail to elicit sufficient T cell help for the activation of  $\text{K}^{\text{bm6}}$ - or  $\text{Qa-1}^a$ -specific  $\text{T}_{\text{eff}}$  cells. However, an alternative possibility is that the response to  $\text{K}^{\text{bm1}}$  allodeterminants generates cells capable of down regulating the response to third-party alloantigens. To test this possibility, we assessed whether mice that had retained indicator  $\text{bm6}$  skin grafts despite rejecting inducer  $(\text{bm6} \times \text{bm1})\text{F}_1$  grafts could subsequently reject these indicator grafts if provided with a proven source of T cell help. Therefore, we engrafted B6 mice with

TABLE I  
*Inability of K<sup>bm1</sup> Allodeterminants to Function as In Vivo Helper Determinants for the Rejection of K<sup>bm6</sup> Disparate Skin Grafts*

Group	Host	Upper (inducer) graft			Lower (indicator) graft			p value†
		Strain	Antigenic disparity	MST*	Strain	Antigenic disparity	MST	
1	B6	bm6	K <sup>bm6</sup>	>60	bm6	K <sup>bm6</sup>	>60	4/21
2	B6	bm6 × bm12	K <sup>bm6</sup> + I <sup>bm12</sup>	14	bm6	K <sup>bm6</sup>	16	23/26
3	B6	B10 × bm12	I <sup>bm12</sup>	9	bm6	K <sup>bm6</sup>	>60	0/7
4	B6	bm6 × bm1	K <sup>bm6</sup> + K <sup>bm1</sup>	18.5	bm6	K <sup>bm6</sup>	>60	7/30

\* Median survival time.

† Statistical comparison of the rejection rates of indicators skin grafts relative to group 1 as determined by the Breslow Test.

‡ p > 0.05.

TABLE II  
*Inability of K<sup>bm1</sup> Allodeterminants to Function as In Vivo Helper Determinants for the Rejection of Qa-1 Disparate Skin Grafts*

Group	Host	Upper (inducer) graft			Lower (indicator) graft			p value†
		Strain	Antigenic disparity	MST*	Strain	Antigenic disparity	MST	
1	B6	B6.T1a <sup>a</sup>	Qa-1 <sup>a</sup>	>60	B6.T1a <sup>a</sup>	Qa-1 <sup>a</sup>	>60	1/5
2	B6	B6.T1a <sup>a</sup> × bm12	Qa-1 <sup>a</sup> + I <sup>bm12</sup>	10	B6.T1a <sup>a</sup>	Qa-1 <sup>a</sup>	21	5/5
3	B6	B6.T1a <sup>a</sup> × bm1	Qa-1 <sup>a</sup> + K <sup>bm1</sup>	17	B6.T1a <sup>a</sup>	Qa-1 <sup>a</sup>	>60	1/5

\* Median survival time.

† Statistical comparison of the rejection rates of indicator skin grafts relative to group 1 as determined by the Breslow Test.

‡ p > 0.05.

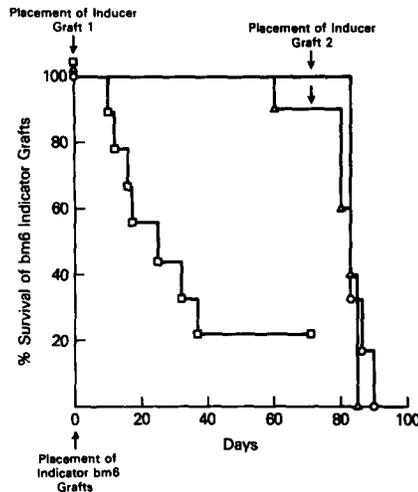


FIGURE 1. Survival of bm6 indicator grafts as a function of inducer graft placement. On day 0, mice were engrafted with bm6 indicator grafts and inducer grafts from  $(\text{bm6} \times \text{bm1})\text{F}_1$  mice (O),  $(\text{bm6} \times \text{bm12})\text{F}_1$  mice (□), or bm6 mice ( $\Delta$ ). Not shown in the graph is that these first inducer grafts were rejected as follows: 9/9 mice rejected  $(\text{bm6} \times \text{bm1})\text{F}_1$  grafts, MST 17 d; 9/9 mice rejected  $(\text{bm6} \times \text{bm12})\text{F}_1$  grafts, MST 14 d; and 1/10 mice rejected bm6 grafts, MST >71 d. On day 71, mice were regrafted with skin from  $(\text{bm6} \times \text{bm12})\text{F}_1$  mice as a second inducer graft. Rejection of the original bm6 indicator grafts is shown as a function of time in days.

$(\text{bm6} \times \text{bm1})\text{F}_1$  inducer and bm6 indicator grafts. As seen in Fig. 1, mice engrafted with inducer grafts expressing both bm6 and bm1 determinants failed to reject their bm6 indicator grafts in 71 d despite rejection of the  $(\text{bm6} \times \text{bm1})\text{F}_1$  inducer grafts (median survival time (MST) 17 d, data not shown). To test whether mice engrafted with  $(\text{bm6} \times \text{bm1})\text{F}_1$  inducer grafts were consequently rendered incapable of rejecting bm6 indicator grafts, we regrafted them on day 71 with a second inducer graft. Mice engrafted with  $(\text{bm6} \times \text{bm12})\text{F}_1$  skin as a second inducer graft rejected their original bm6 indicator grafts with a MST of 12 d (Fig. 1). This rejection rate of bm6 indicator grafts was similar to that induced by  $(\text{bm6} \times \text{bm12})\text{F}_1$  inducer grafts on naive mice (MST of 12 d). Thus, inducer skin grafts expressing MHC class I  $\text{K}^{\text{bm1}}$  allodeterminants as well as  $\text{K}^{\text{bm6}}$  target determinants did not generate functionally detectable  $\text{K}^{\text{bm6}}$ -specific suppression.

*Failure of  $\text{Lyt-2}^+$  T Cells to Generate Help for In Vivo Rejection of Third-party Allografts.* We next considered the possibility that  $\text{L3T4}^+$  T cells present in normal engrafted mice might have interfered with the helper function of MHC class I-allo-specific T cells. Consistent with such a possibility is the observation that mice reconstituted with isolated populations of  $\text{Lyt-2}^+$  T cells frequently reject class I disparate skin allografts more rapidly than mice reconstituted with T cell populations containing both  $\text{Lyt-2}^+$  and  $\text{L3T4}^+$  T cells (3). To examine the ability of MHC class I  $\text{K}^{\text{bm1}}$  allodeterminants to function as in vivo helper determinants in the absence of  $\text{L3T4}^+$  T cells, we adoptively transferred unfractionated or  $\text{L3T4}^-$  spleen cells into B10 nude mice that had been engrafted with both inducer and indicator skin allografts (Table III). We used the male H-Y antigen as the target antigen in this experiment because effector cells mediating the anti-H-Y rejection response are  $\text{Lyt-2}^+$  and require additional help (1, 19). Indeed, mice reconstituted with isolated  $\text{Lyt-2}^+$  T cells failed to reject H-Y disparate B6 male indicator grafts (Table III, group 1). More importantly, such mice failed to reject H-Y disparate indicator grafts even when engrafted with inducer grafts expressing both the H-Y target antigens and  $\text{K}^{\text{bm1}}$  allodeterminants (Table III, group 2). These mice did, however, reject the  $\text{K}^{\text{bm1}}$ -

TABLE III  
 Failure of MHC Class I-allospecific Lyt-2<sup>+</sup> T Cells to Generate Help for the Rejection of H-Y Disparate Skin Grafts

Group	Host	Sex	Reconstituting H-2 <sup>b</sup> spleen cells <sup>†</sup>	Upper (inducer) graft			Lower (indicator) graft				
				Strain	Antigenic disparity	MST*	Fraction rejecting	Strain	Antigenic disparity	MST	Fraction rejecting
1	B10 nu/nu	F	Lyt-2 <sup>+</sup>	B6 M	H-Y	>61	0/5	B6 M	H-Y	>61	0/5
2	B10 nu/nu	F	Lyt-2 <sup>+</sup>	bm1 M	K <sup>bm1</sup> + H-Y	17.5	8/8	B6 M	H-Y	>61	0/8
3	B10 nu/nu	F	L3T4 <sup>+</sup> + Lyt-2 <sup>+</sup>	B6 M	H-Y	42	5/6	B6 M	H-Y	36	4/6
4	B10 nu/nu	F	L3T4 <sup>+</sup> + Lyt-2 <sup>+</sup>	bm1 M	K <sup>bm1</sup> + H-Y	17	8/8	B6 M	H-Y	38	8/8

\* Median survival time.

† H-2<sup>b</sup> female nude mice were engrafted with inducer and indicator grafts as indicated on day 0 and reconstituted with H-2<sup>b</sup> female spleen cells on day 1. Lyt-2<sup>+</sup> spleen cells were H-2<sup>b</sup> spleen cells depleted of L3T4<sup>+</sup> cells by treatment with anti-L3T4 mAb and C'. L3T4<sup>+</sup> + Lyt-2<sup>+</sup> spleen cells were unfractionated H-2<sup>b</sup> spleen cells.

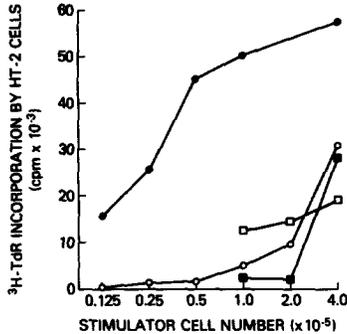


FIGURE 2. Response of  $L3T4^+$ ,  $Lyt-2^+$  lymphokine-secreting T cells from B6 mice engrafted with bm1 skin allografts. B6 responder spleen cells were depleted of adherent cells by passage over a Sephadex-G10 column and then treated with anti- $L3T4$  mAb and  $C'$  to deplete  $L3T4^+$  T cells. Responder cells from naive B6 mice (*open symbols*) or B6 mice that had been engrafted with and had rejected bm1 skin allografts (*closed symbols*) were cultured with spleen stimulator cells from either bm1 (○, ●) or H-2D<sup>a</sup> expressing B10.QBR (□, ■) mice. Each response culture contained anti-IL-2-R mAb (0.01% of 7D4 ascites) to inhibit IL-2 consumption during the culture period. On day 4, the supernatant from each culture was assayed for its IL-2 content on HT-2 cells.

bearing inducer grafts (Table III, group 2), confirming that mice reconstituted with isolated  $Lyt-2^+$  T cells were capable of rejecting skin allografts bearing MHC class I allodeterminants (1-3). Thus, despite the absence of  $L3T4^+$  T cells,  $Lyt-2^+$  anti- $K^{bm1}$  T cells failed to provide help for the activation of anti-H-Y effector cells, even as they were rejecting the  $K^{bm1}$ -bearing inducer grafts.  $Lyt-2^+$  anti-H-Y effector cells were capable of rejecting the H-Y indicator grafts when help was provided by  $L3T4^+$   $T_h$  cells, whether or not  $K^{bm1}$  allodeterminants were present (Table III, groups 3, 4). Finally, it should be pointed out that  $L3T4^+$  T cells function as helper cells in anti-H-Y responses and are themselves incapable of rejecting the H-Y indicator grafts (1, 19).

*Lyt-2<sup>+</sup> Class I-specific T<sub>h</sub> Are Primed by Skin Grafts Expressing Allogeneic Class I Determinants.* Because  $K^{bm1}$  class I allodeterminants failed to activate  $T_h$  cells able to induce the rejection of third-party skin allografts, it was necessary to ascertain that MHC class I-allo-specific  $T_h$  cells had in fact recognized and responded to  $K^{bm1}$  class I allodeterminants expressed by bm1 inducer skin grafts. To assess the effect, if any, of bm1 skin allografts on anti- $K^{bm1}$  lymphokine-secreting  $Lyt-2^+$   $T_h$  cells, we compared the in vitro IL-2 secretion responses of  $Lyt-2^+$   $T_h$  cells from naive mice and from mice that had been engrafted with, and had rejected, bm1 tailskin allografts. Anti-IL-2-R mAb was added to the in vitro response cultures to block consumption of IL-2 during the response period, thereby permitting an accurate and sensitive measure of the IL-2 produced (14, 17). As can be seen in Fig. 2,  $Lyt-2^+$  T cells from mice that had been engrafted with bm1 skin allografts exhibited a marked shift in their anti- $K^{bm1}$  dose/response curve in that they required ~16-fold fewer stimulator cells for comparable IL-2 production than did  $Lyt-2^+$  T cells from naive mice. In addition, the response of  $Lyt-2^+$  T cells from bm1-engrafted mice was greater at each stimulator cell dose than that of  $Lyt-2^+$  T cells from naive mice. That these effects were specific for  $K^{bm1}$  allodeterminants expressed by the skin allograft is shown by the failure of  $Lyt-2^+$   $T_h$  from bm1-engrafted mice to manifest similarly increased responses to third-party class I H-2D<sup>a</sup> allodeterminants (Fig. 2). Thus, anti- $K^{bm1}$ ,  $Lyt-2^+$  lymphokine-secreting  $T_h$  are specifically primed by  $K^{bm1}$  class I disparate skin allografts, demonstrating that  $Lyt-2^+$  lymphokine-secreting  $T_h$  cells do recognize and respond in vivo to class I allodeterminants expressed on skin.

*Class I-specific T Cells Consume the IL-2 They Produce.* Having determined that  $Lyt-2^+$  lymphokine-secreting  $T_h$  cells do respond to MHC class I allodeterminants ex-

TABLE IV  
*Production and Consumption of IL-2 by MHC Class I-reactive T Cells*

Exp.	G10 passed responder	Stimulator cells	Stimulator cell antigen	Blocking mAb*	<sup>3</sup> H]TdR incorporation by HT-2 cells	
					No mAb added	Anti-IL-2-R added†
<i>cpm × 10<sup>-3</sup></i>						
1	B6	Media	-	-	1.2	3.8
	B6	B6	self	-	3.3	7.9
	B6	bm1	K <sup>bm1</sup>	-	4.4	47.9
	B6	bm12	I <sup>bm12</sup>	-	20.8	41.2
2	B10	Media	-	-	0.2	0.6
	B10	B10	self	-	7.7	14.5
	B10	bm1	K <sup>bm1</sup>	-	2.6	42.7
	B10	Media	-	anti-IA <sup>b</sup>	0.2	0.5
	B10	B10	self	anti-IA <sup>b</sup>	0.1	0.4
	B10	bm1	K <sup>bm1</sup>	anti-IA <sup>b</sup>	4.8	29.0

\* M5/114 mAb, 25% culture supernatant (vol/vol).

† 7D4, mAb, 0.01% ascites (vol/vol).

pressed on skin allografts, it was unclear why MHC class I-disparate inducer skin allografts did not activate T<sub>h</sub> cells able to induce rejection of third-party indicator skin grafts, as did MHC class II-disparate inducer skin allografts. One possibility was that lymphokine-secreting T<sub>h</sub> cells specific for MHC class I or MHC class II alloantigens differed in the amount of lymphokines that they themselves consumed and so differed in the amount of helper lymphokines that they could provide to third-party T<sub>eff</sub> cells. To compare the amount of soluble IL-2 produced and consumed by T cells upon recognition of MHC class I and MHC class II allodeterminants, we measured IL-2 present in supernatants of response cultures under conditions in which consumption of IL-2 by responder cells either was permitted or was blocked by the presence of anti-IL-2-R mAb (14, 17). It has been previously documented that anti-IL-2-R mAb fails to stimulate the secretion of IL-2 by populations of MHC class I-specific lymphokine-secreting T<sub>h</sub> cells (17). It can be seen in Table IV that the activity of soluble IL-2 present in the supernatants of response cultures stimulated by recognition of K<sup>bm1</sup> class I allodeterminants was affected dramatically by the presence or absence of anti-IL-2-R mAb. Indeed, in the absence of anti-IL-2-R mAb, essentially all the IL-2 produced in response to recognition of K<sup>bm1</sup> class I allodeterminants was consumed, whereas that was not the case in response to I<sup>bm12</sup> class II allodeterminants (Table IV, exp. 1). In a second experiment, we added anti-I-A<sup>b</sup> mAb to the response cultures to block activation of T<sub>h</sub> cells recognizing K<sup>bm1</sup> class I allodeterminants in the context of I-A<sup>b</sup> (17, 20), so that we could focus exclusively on anti-K<sup>bm1</sup> class I-restricted T<sub>h</sub> cells (Table IV, exp. 2). It can be seen that either in the presence or absence of anti-I-A<sup>b</sup> mAb, anti-bm1 T cells consumed most of the IL-2 they produced (Table IV, exp. 2). That the anti-I-A<sup>b</sup> mAb was effective in these cultures is indicated by its blockade of the class II-restricted anti-self response (Table IV, exp. 2). The ability of anti-I-A<sup>b</sup> mAb to completely block L3T4<sup>+</sup> class I-specific lymphokine-secreting T<sub>h</sub> has been previously documented

(17). Thus, in contrast to  $I^{bm12}$  class II-allospecific T cells,  $K^{bm1}$  class I-allospecific T cells consume essentially all the IL-2 produced.

### Discussion

The present study demonstrates that, unlike recognition of MHC class II allodeterminants, recognition of MHC class I allodeterminants expressed on skin grafts fails to generate in vivo help for the rejection of third-party skin allografts. That MHC class I-allospecific  $T_h$  cells recognize and respond to MHC class I allodeterminants expressed on skin grafts was shown by the fact that engraftment of mice with class I-disparate skin specifically primed lymphokine-secreting class I-allospecific  $T_h$  cells. However, measurement of the IL-2 produced by class I-allospecific  $T_h$  cells in response to MHC class I allodeterminants revealed that the responding cells consumed nearly all the IL-2 that was produced. Taken together, these data are most consistent with the concept that rejection of skin grafts expressing only class I allodeterminants is mediated by class I-allospecific dual-function  $T_h/T_{eff}$  cells that secrete IL-2 when activated, but preferentially consume most of the helper lymphokines they secrete.

We have previously shown that the ability to reject skin allografts is not unique to a specialized subset of T cells of a given phenotype or a given function, since allograft rejection can result from interactions between distinct populations of lymphokine-secreting  $T_h$  cells and lymphokine-responsive  $T_{eff}$  cells that are of different Lyt phenotypes (1). Indeed, the interacting  $T_h$  and  $T_{eff}$  cells can even express distinct antigen specificities, as is the case with  $I^{bm12}$ -specific  $T_h$  cells activating  $K^{bm6}$ -specific T effector cells to reject  $K^{bm6}$ -disparate skin grafts (1). Thus, interaction with a distinct population of  $T_h$  cells is one mechanism by which MHC class I-allospecific  $T_{eff}$  cells can be activated to reject skin allografts expressing MHC class I allodeterminants. However, these results did not address the possibility that a second rejection mechanism involving a single subpopulation of dual-function T cells that both secrete helper lymphokines and differentiate into cytolytic effector cells might also mediate graft rejection responses without any further cell-cell interaction requirements. T cell populations containing dual-function cells would be read out as containing both lymphokine-secreting  $T_h$  cells and lymphokine-responsive  $T_{eff}$  cells and so would be expected to reject skin allografts of appropriate specificities. Dual-function cells were especially likely to participate in rejection of class I-disparate skin allografts because: (a) rejection of class I-disparate skin allografts does not require interactions between phenotypically distinct T cell populations but can be mediated by a single population of Lyt-2<sup>+</sup> T cells; and (b) T cell clones with dual function have been shown to be capable of rejecting MHC class I-disparate pancreatic islet grafts (21) and Lyt-2<sup>+</sup> class I-restricted dual-function cells have been shown capable of rejecting allogeneic tumor grafts (22). However, it is not known whether T cells with dual-function capability actually play any role in in vivo allograft rejection responses of normal mice.

To distinguish between the possibilities that class I-disparate skin allografts activate distinct populations of helper lymphokine-secreting  $T_h$  cells and lymphokine-responsive  $T_{eff}$  cells vs. activating a single population of dual-function T cells that produce their own helper lymphokines, we assessed the ability of T cells reactive against skin class I allodeterminants to provide help for the rejection of third-party

skin allografts, in a manner analogous to that provided by MHC class II-allo-specific  $T_h$  cells. We specifically selected  $K^{bm1}$  as the stimulatory MHC class I allodeterminant for these experiments, because the frequency of anti- $K^{bm1}$  lymphokine-secreting  $T_h$  cells is higher than for any other class I specificity we have examined (3, 23), and because the quantity of helper lymphokine produced in anti- $K^{bm1}$  responses is greater than for any other class I-allo-specific response we have examined (23). Even so, we observed a marked disparity in the relative abilities of anti- $K^{bm1}$  vs. anti- $I^{bm12}$   $T$  cells to provide help for  $K^{bm6}$ - or  $Qa-1^a$ -specific  $T_{eff}$  cell populations. The failure of anti- $K^{bm1}$  class I-allo-specific  $T$  cells to provide help for third-party effector cell populations could have a basis in quantitative or qualitative factors.

It was conceivable that the precursor frequency of  $K^{bm1}$ -specific  $T_h$  cells, as opposed to that of  $I^{bm12}$ -specific  $T_h$  cells, might be insufficient to provide help for third-party  $T_{eff}$  cells. However, previous studies have documented that the frequencies of anti- $K^{bm1}$  and anti- $I^{bm12}$  lymphokine-secreting  $T_h$  cell precursors are comparable (23, 24).

It was conceivable that MHC class I-allo-specific  $T_h$  cells, while present in the engrafted host, did not respond to MHC class I allodeterminants expressed on skin cells. However, when we compared the responses of class I-allo-specific IL-2-secreting  $T_h$  cells from mice that had been engrafted with  $K^{bm1}$  skin allografts with responses of cells from naive mice, we found that anti- $K^{bm1}$  lymphokine-secreting  $T$  cells were clearly primed by engraftment with  $bm1$  skin. Thus, class I-allo-specific  $T_h$  cells do recognize and respond to MHC class I allodeterminants expressed on skin allografts.

Finally, it was conceivable that class I-allo-specific  $T_h$  cells might secrete very little lymphokine on a cell for cell basis, or alternatively, that class I-allo-responsive  $T$  cells might consume greater amounts of lymphokine than do class II-allo-responsive  $T$  cells. We addressed these points by stimulating anti-class I lymphokine responses in vitro and comparing the amount of soluble lymphokine secreted into the media when the consumption of these factors was either blocked or not. It was clear that the anti-class I lymphokine response was substantial when lymphokine consumption was blocked, but that when consumption was not blocked, the class I-reactive  $T$  cells consumed nearly all of the soluble lymphokine produced, leaving little soluble lymphokine in the medium. Thus, we think the failure of class I allodeterminants to provide help in vivo for activating third-party effector cells is due to the fact that lymphokine-secreting  $T$  cells reactive to class I allodeterminants on skin grafts consume most of the helper lymphokines they produce. This property is precisely that expected of dual-function  $T_h/T_{eff}$  cells and so indicates that rejection of skin allografts expressing only class I disparities can be mediated by dual-function  $T$  cells that preferentially consume the helper factors they secrete. This conclusion is entirely consistent with previous work demonstrating that only  $T$  cell populations containing both  $T_h$  and  $T_{eff}$  cellular functions are able to mediate graft rejection (1). Thus,  $T_h$  and  $T_{eff}$  cellular functions may be generated by distinct, interacting cellular populations, or may be generated by a single population of dual-function cells.

The observation that MHC class I allodeterminants fail to trigger  $T$  cell help for the in vivo activation of  $T_{eff}$  cells with third-party specificities sharply contrasts with their ability to do so in vitro (25). Dual-function  $T$  cells appear similar to conventional  $T_h$  cells in that they secrete soluble helper lymphokines. Dual-function  $T$  cells are also similar to conventional  $T_{eff}$  cells in that they bind soluble lymphokine to

specific receptors on the cell surface, and this binding is susceptible to blockade by anti-receptor antibody. Thus, the primary advantage that dual-function T cells have over conventional helper-dependent  $T_{\text{eff}}$  cells is that the dual-function cell is necessarily more proximal to the secreted lymphokines. We think that the proximity advantage is largely negated in vitro where T cell subpopulations are artificially brought together. In contrast, proximity advantages are likely to be of critical importance in vivo, where T cells have to migrate to the reaction site, particularly when secreted helper lymphokine is present in limiting amounts.

Taken together, these data demonstrate that there are two  $T_{\text{eff}}$  cell populations able to mediate the rejection of MHC class I-disparate skin grafts. There are class I-specific effector cells, such as anti- $K^{\text{bm6}}$  effector cells, that are themselves unable to secrete lymphokine but can use exogenous T cell help to become activated and reject  $\text{bm6}$  skin allografts. In addition, there are class I-specific effector cells, such as anti- $K^{\text{bm1}}$  effector cells, that produce and consume their own  $T_{\text{h}}$  factors in response to skin grafts expressing  $K^{\text{bm1}}$  allodeterminants. Thus, MHC class I-disparate grafts can be rejected by two cellular mechanisms depending on the ability of the  $T_{\text{eff}}$  cell population to secrete lymphokine in response to class I alloantigen: (a) a dual-function cell mechanism and (b) a mechanism requiring an interaction between distinct populations of lymphokine-secreting  $T_{\text{h}}$  cells and lymphokine-responsive  $T_{\text{eff}}$  cells.

### Summary

The present study further characterizes the cellular mechanisms involved in the in vivo rejection of MHC class I-disparate skin allografts. Previously, we demonstrated that class I-specific rejection responses could result from collaborations between distinct populations of lymphokine-secreting T helper ( $T_{\text{h}}$ ) and lymphokine-responsive T effector ( $T_{\text{eff}}$ ) cells. In the present study, we have assessed the possibility that class I-specific rejection responses could also result from a second cellular mechanism involving a single population of dual-function  $T_{\text{h}}/T_{\text{eff}}$  cells that would not have any further requirement for cell-cell collaboration. Our experimental strategy was to determine the ability of MHC class I-allo-specific T cells, in response to class I allodeterminants expressed on skin grafts, to provide help in vivo for activation of helper-dependent  $T_{\text{eff}}$  cells. We found that class I anti- $K^{\text{bm1}}$ -allo-specific T cells would reject  $\text{bm1}$  skin allografts, but would not generate help for the activation of helper-dependent effector cells that were specific for third-party skin allografts (e.g., grafts expressing  $K^{\text{bm6}}$ ,  $\text{Qa1}^{\text{a}}$ , or H-Y allodeterminants). This failure of anti- $K^{\text{bm1}}$  T cells to provide help in response to  $\text{bm1}$  skin allografts was not due to an inability of lymphokine-secreting anti- $K^{\text{bm1}}$   $T_{\text{h}}$  cells to recognize and respond in vivo to  $K^{\text{bm1}}$  allodeterminants expressed on skin, since lymphokine-secreting anti- $K^{\text{bm1}}$   $T_{\text{h}}$  cells were specifically primed in animals engrafted with  $\text{bm1}$  skin allografts. Nor was any evidence found that this failure was due to active suppression of anti- $K^{\text{bm1}}$  helper activity. Rather, we found that anti- $K^{\text{bm1}}$  T cells consumed nearly all of the helper factors they secreted. Taken together, these results are most consistent with the in vivo activity of dual-function  $T_{\text{h}}/T_{\text{eff}}$  cells that consume the lymphokines they secrete.

Thus, this study demonstrates that MHC class I-disparate skin allografts can be

rejected by two mechanisms, depending on the ability of the allospecific T<sub>eff</sub> cell to secrete helper lymphokines. MHC class I-disparate grafts can be rejected by (a) class I-allospecific T<sub>eff</sub> cells that are unable to produce lymphokine but are responsive to exogenous T cell help; and (b) class I-allospecific dual-function T<sub>h</sub>/T<sub>eff</sub> cells that are able to both produce and consume soluble lymphokine.

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### References

1. Rosenberg, A. S., T. Mizuochi, S. O. Sharrow, and A. Singer. 1987. Phenotype, specificity, and function of T cell subsets and T cell interactions involved in skin allograft rejection. *J. Exp. Med.* 165:1296.
2. Sprent, J., M. Schaefer, D. Lo, and R. Korngold. 1986. Properties of purified T cell subsets II. In vivo responses to class I vs. class II H-2 differences. *J. Exp. Med.* 163:998.
3. Rosenberg, A. S., T. Mizuochi, and A. Singer. 1986. Analysis of T-cell subsets in rejection of K<sup>b</sup> mutant skin allografts differing at class I MHC. *Nature (Lond.)* 322:829.
4. Widmer, M. B., and F. H. Bach. 1981. Antigen-driven helper cell-independent cloned cytotoxic T lymphocytes. *Nature (Lond.)* 294:750.
5. Roopenian, D. C., M. B. Widmer, G. C. Orosz, and F. H. Bach. 1983. Helper cell-independent cytolytic T lymphocytes specific for a minor histocompatibility antigen. *J. Immunol.* 130:542.
6. Von Boehmer, H., and K. Turton. 1983. Autonomously proliferating K/D-restricted cytolytic T cell clones. *Eur. J. Immunol.* 13:176.
7. Boyse, E. A., L. J. Old, and E. Stockert. 1965. The T1 (thymus leukemia) antigen: a review. *Int. Symp. Immunopathol., 4<sup>th</sup>, Monte Carlo*. P. Grabar and P. Miescher, editors. Grune & Stratton Inc., New York. 4:23-40.
8. Kohn, H. I., J. Klein, R. W. Melvold, S. G. Nathenson, D. Pious, and D. C. Shreffler. 1978. The first H-2 mutant workshop. *Immunogenetics.* 7:279.
9. McKenzie, I. F. C., G. M. Morgan, M. S. Sandrin, M. M. Michaelides, R. W. Melvold, and H. I. Kohn. 1979. B6.CH-2<sup>bm12</sup>: a new H-2 mutation in the I region in the mouse. *J. Exp. Med.* 150:1323.
10. Melief, C. J. M., R. S. Schwartz, H. I. Kohn, and R. W. Melvold. 1975. Dermal histocompatibility and in vitro lymphocyte reactions of three new H-2 mutants. *Immunogenetics.* 2:337.
11. Dialynas, D. P., Z. S. Quan, K. A. Wall, A. Pierres, J. Quintans, M. R. Loken, M. Pierres, and F. W. Fitch. 1984. Characterization of the murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to human Leu3/T4 molecule. *J. Immunol.* 131:2445.
12. Ceredig, R., J. W. Lowenthal, M. Nabholz, and H. R. MacDonald. 1985. Expression of interleukin-2 receptors as a differentiation marker on intrathymic stem cells. *Nature (Lond.)* 314:98.
13. Bhattacharya, A., M. E. Dorf, and T. A. Springer. 1981. A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: evidence for I region gene duplication. *J. Immunol.* 127:2448.
14. Malek, T. R., R. J. Robb, and E. M. Shevach. 1983. Identification and initial characterization of a rat monoclonal antibody reactive with the murine interleukin 2 receptor-ligand complex. *Proc. Natl. Acad. Sci. USA.* 80:5694.

15. Billingham, R. E., and P. B. Medawar. 1951. The technique of free skin grafting in mammals. *J. Exp. Biol.* 28:385.
16. Hathcock, K. S., A. Singer, and R. J. Hodes. 1981. Passage over Sephadex G-10 columns. *In* Manual of Macrophage Methodology: Collection, Characterization, and Function. H. B. Herschowitz, H. T. Holden, J. A. Bellanti, and A. Ghuffer, editors. Marcel Dekker, Inc., New York. 127-133.
17. Mizuochi, T., S. Ono, T. R. Malek, and A. Singer. 1986. Characterization of two distinct primary T cell populations that secrete IL-2 upon recognition of class I or class II major histocompatibility antigens. *J. Exp. Med.* 163:603.
18. Watson, J. 1979. Continuous proliferation of murine antigen-specific helper T lymphocytes in culture. *J. Exp. Med.* 150:1510.
19. Boog, C. J. P., W. M. Kast, H. T. M. Timmers, J. Boes, L. P. deWaal, and C. J. M. Melief. 1985. Abolition of specific immune response defect by immunization with dendritic cells. *Nature (Lond.)* 318:59.
20. Golding, H., T. Mizuochi, S. A. McCarthy, C. A. Cleveland, and A. Singer. 1987. Relationship among function, phenotype, and specificity in primary allospecific T cell populations: identification of phenotypically identical but functionally distinct primary T cell subsets that differ in their recognition of MHC class I and Class II allodeterminants. *J. Immunol.* 138:10.
21. Isakov, N., L. N. Biel, and F. H. Bach. 1985. Induction of Class I antigen-disparate pancreatic islet graft rejection by in vivo administration of cloned helper cell-independent cytolytic T lymphocytes. *Transplant. Proc.* 17:727.
22. Engers, H. D., A. L. Glasebrook, and G. D. Sorenson. 1982. Allogeneic tumor rejection induced by the intravenous injection of Lyt-2<sup>+</sup> cytolytic T lymphocyte clones. *J. Exp. Med.* 156:1280.
23. Mizuochi, T., T. I. Munitz, S. A. McCarthy, P. M. Andrysiak, J. Kung, R. E. Gress, and A. Singer. 1986. Differential helper and effector responses of Lyt2<sup>+</sup> T cells to H-2K<sup>b</sup> mutant (K<sup>bm</sup>) determinants and the appearance of thymic influence on anti-K<sup>bm</sup> CTL responsiveness. *J. Immunol.* 137:2740.
24. Heeg, K., C. Steeg, J. Schmitt, and H. Wagner. 1987. Frequency analysis of class I MHC-reactive Lyt-2<sup>+</sup> and class II MHC-reactive L3T4<sup>+</sup> IL2-secreting T lymphocytes. *J. Immunol.* 138:4121.
25. Mizuochi, T., H. Golding, A. S. Rosenberg, L. H. Glimcher, T. R. Malek, and A. Singer. 1985. Both L3T4<sup>+</sup> and Lyt-2<sup>+</sup> helper T cells initiate cytotoxic T lymphocyte responses against allogeneic major histocompatibility antigens but not against trinitrophenyl-modified self. *J. Exp. Med.* 162:427.