The Journal of Immunology

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J Immunol 2007; 178:3558-3565; ;
doi: 10.4049/jimmunol.178.6.3558
http://www.jimmunol.org/content/178/6/3558

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Natural Regulation of Immunity to Minor Histocompatibility Antigens[1]

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MHC-matched hemopoietic stem cell transplantation is commonly used for the treatment of some forms of leukemia. Conditioning regimens before transplant act to reduce the burden of leukemic cells and the graft-vs-leukemia (GvL) effect can eliminate residual disease. The GvL effect results largely from the recognition of minor histocompatibility Ags by donor T cells on recipient tissues. These Ags are generally widely expressed and also provoke graft-vs-host (GvH) disease. Manipulation of immunity to promote GvL while curtailing GvH would greatly improve clinical outcome. To develop strategies that may achieve this, the parameters which control immunity to minor histocompatibility Ags need to be defined. In this study, we have analyzed responses to the mouse HY minor histocompatibility Ag using hemopoietic cell and skin grafts as surrogate GvL and GvH targets, respectively. We show that natural regulation of CD8 T cell responses to HY operates at multiple levels. First, CD4 T cell help is required for primary CD8 responses directed at hemopoietic cells. However, although CD4 T cells of $H2^k$ mouse strains recognize HY, they provide ineffective help associated with a proportion of recipients developing tolerance. This was further investigated using TCR-transgenic mice which revealed $H2^k$-restricted HY-specific CD4 T cells are highly susceptible to regulation by $CD25^+$ regulatory T cells which expand in tolerant recipients. A second level of regulation, operating in the context of skin grafts, involves direct inhibition of CD8 T cell responses by CD94/NKG2 engagement of the nonclassical MHC class I molecule Qa1. ***The Journal of Immunology,*** **2007, 178: 3558–3565.**

T cell responses directed at minor histocompatibility Ags are responsible for the rejection of MHC-matched transplants. Bone marrow transplantation between MHC-matched siblings is a standard procedure for the treatment of hemological malignancy and responses to minor histocompatibility Ags elicit the major complications of host-vs-graft and graft-vs-host (GvH)[3] disease. The GvH response also targets recipient hemopoietic cells including residual leukemic cells (1).

Adoptive transfer of minor histocompatibility-specific CD8 T cells offers an approach for the eradication of hemopoietic malignancy (2, 3). For this approach to be clinically applied, a fuller understanding of immunity to minor histocompatibility Ags is needed. In particular, the identification of T cell populations able to target hemopoietic tissue while not promoting GvH disease is a key objective. Minor histocompatibility Ags expressed specifically on hemopoietic lineages are attractive targets (4), although the majority of minor Ags are broadly expressed. Despite widespread expression, immunity to single minor histocompatibility Ags can specifically eliminate leukemic and solid tumor cells without inducing GvH (5, 6).

Although many minor histocompatibility Ags have been characterized in mice and humans (7), relatively little is understood about natural regulation of immunity to minor histocompatibility Ags. Although CD8 T cells alone can reject grafts expressing multiple minor histocompatibility Ags (8), genetic dissection of single minor histocompatibility loci has identified the presence of both MHC class I- and class II-restricted epitopes, indicating a requirement for cooperation between the CD4 and CD8 T cell subsets to generate effective responses (9, 10). Indeed, presentation of MHC class I-restricted epitopes in the absence of CD4 T cell help, or depletion of either the CD4 or the CD8 recipient T cell subset, can lead to the induction of nonresponsiveness (10, 11). Although CD8 T cell priming and expansion can occur in the absence of CD4 T cell help, the resulting cells can be compromised in their capacity to differentiate into memory cells (12). Strategies for inducing tolerance to minor histocompatibility Ags have focused mainly on targeting CD4 Th cells. Deletional and nondeletional approaches to controlling the helper response can be effective. Nondeletional tolerance often involves the induction of regulatory T cells (Treg) which can arise from nonregulatory cells (13). It is also clear that naturally occurring Tregs can moderate immunity directed to minor histocompatibility Ags in TCR-transgenic mice (14). The importance of naturally occurring $CD25^+$CD4 Treg cells in regulating minor histocompatibility responses has not, however, been explored. T cells also use intrinsic mechanisms to control their signaling, expansion, and differentiation into memory cells. Here, we have also explored the function of inhibitory signaling via the CD94/NKG2 heterodimer on regulating CD8 T cell immunity to minor histocompatibility Ags.

We have taken advantage of the detailed molecular description of the mouse HY minor histocompatibility Ag (7) and the availability of mouse strains expressing different sets of HY epitopes to

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Received for publication September 28, 2006. Accepted for publication December 26, 2006.

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[1] This work was funded by the U.K. Medical Research Council.

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[3] Abbreviations used in this paper: GvH, graft-vs-host; Treg, regulatory T cell; β_2m, β_2-microglobulin; KO, knockout; WT, wild type.

evaluate factors controlling immunity to hemopoietic and skin grafts. The HY minor histocompatibility Ag is particularly amenable to analysis because the epitopes are encoded by genes on the Y chromosome and, in mice, these map to the Δ*Sxr* deletion interval (15), and in humans to a syntenic region on Yq. Further, most human and mouse HY peptide epitopes derive from the same set of genes: *Uty/UTY* and *Smcy/SMCY* for MHC class I and *Dby/DBY* for class II epitopes. In mice, responsiveness to HY varies widely between strains; females of $H2^b$ haplotype are strong responders capable of rejecting primary syngeneic male skin grafts, while females of $H2^d$ strains are resolute nonresponders (16). In contrast, $H2^k$ strains are weak responders to HY and either fail to reject male skin grafts or do so only following immunization.

The weak, variable response of $H2^k$ strain females to male skin grafts is surprising given the presentation and recognition of Y chromosome-encoded peptides on MHC class I (HYK^k*Smcy*; HYD^k*Smcy*) and MHC class II (HYE^k*Dby*) (17–19). Indeed, in F_1 $H2^{bxk}$ females, the HYD^k*Smcy* response is immunodominant over both HYK^k*Smcy* and HYD^b*Uty*, the dominant $H2^b$-restricted class I epitope (19). Here, the basis for the weak immune response to HY in the context of the $H2^k$ haplotype has been dissected and found to be multifactorial.

First, the primary expansion of the immunodominant HYD^k*Smcy* CD8 T cell response is severely compromised despite the presence of HY-specific CD4 Th cells recognizing HYE^k*Dby*. This is associated with sustained nonresponsiveness to successive hemopoietic cell grafts in a proportion of recipients. Strikingly, HYE^k-specific CD4 T cells are remarkably sensitive to regulation in comparison with HYA^b-specific CD4 T cells. A further component of the failure of $H2^k$ strains to reject male skin grafts is that despite the immunodominance of the HYD^k*Smcy* CD8 T cell response, it is relatively ineffective at rejecting skin. Finally, we show that rejection of skin by HY-specific CD8 T cells is moderated by inhibitory signaling through the NKG2/CD94 receptor following engagement of its ligand Qa1/Qdm.

Materials and Methods

Mice

C57BL/6J (B6), CBA/Ca (CBA), BALB/cOla (BALB/c), B10.A(5R), and (C57BL/6J × CBA/Ca)F_1 mice were purchased from Harlan Olac. TCR-transgenic strain Marilyn (20) was provided by Dr. O. Lantz. (Institute Curie, Paris, France). β_2-Microglobulin (β_2m) knockout (KO) (B6 background) was provided by The Jackson Laboratory. B10.GD, NOD, (B10.A(5R) × CBA)F_1 and the TCR-transgenic line A1 (21) were bred at the Biological Services Unit at the Medical Research Council Clinical Sciences Centre. B6 knockout strains for $H2K^b$, D^b, and K^bD^d (22) were maintained at the Pasteur Institute. Mice were used at age 6–10 wk. Table I summarizes the relevant peptide epitopes for the strains used in this study. All animal procedures have been approved by the Institutional Review Committee and the Home Office.

ELISPOT assay

These were performed as described previously (23).

Immunization

Spleen cells were prepared by gentle teasing of dissected spleen. Cells were then passed through a cell strainer, washed twice in balanced salt solution, and resuspended in PBS for i.p. or i.v. (tail vein) injection.

In vivo cytotoxicity assay

Female and male B6 spleen cells (2×10^7/ml in PBS) harvested as above were incubated with 0.5 and 5 μM CFSE (Molecular Probes), respectively, at room temperature for 8 min in the dark and FCS added to 20%. After washing, the cells were mixed, resuspended in PBS, and 2×10^7 cells in 200 μl were injected i.v. into each recipient. Peripheral blood was collected from individual mice at 24 h to confirm the initial female:male cell ratio. Further blood samples were taken to monitor specific elimination of male cells. After lysis of RBCs and blockade of FcR, PBLs were stained with anti-CD8-PerCP (BD Pharmingen) and analyzed for CFSE expression by flow cytometry.

Table I. *Mouse strains and relevant peptide epitopes*

	D^k/K^k*Smcy*	D^b*Uty*/*Smcy*	E^k*Dby*	A^b*Dby*	*Qdm*
C57BL/6		+		+	+
CBA, B10.BR	+		+		+
[C57BL/6 × CBA]F_1	+	+	+	+	+
C57BL/6 β2m KO				+	
B10.GD		+			+
B10.A(5R)				+	+
[B10.A(5R) × CBA]F_1	+		+	+	+
A1 TCR transgenic			+		
Marilyn TCR transgenic				+	
C57BL/6 K^b KO		+		+	+
C57BL/6 D^b KO				+	
C57BL/6 K^b/D^b KO				+	

Cell staining and analysis

PBL were prepared by direct collection of tail blood into 200 μl of blood buffer (10 mM EDTA, 100 U of heparin/ml in PBS) followed by addition of 1 ml of red cell lysis buffer (Puregene; RBC lysis solution). Samples were left at room temperature for 15′ and microfuged (4 min, 3500 rpm) and washed twice in PBS. A total of 10^6 peripheral blood cells were stained with anti-CD25, anti-CD4, anti-CD8, anti-NKG2, anti-Vβ6, anti-Vβ8.2 (BD Pharmingen) at 4°C for 30 min and washed in PBS containing 2% FCS (FACS buffer). MHC class I-peptide tetramers (HYD^k*Smcy*RRLRK TLL and HYK^k*Smcy*TENSGKDI) were purchased from Proimmune. Aliquots of 10^6 cells were stained in 50 μl of with 1 μl of tetramer for 10 min at room temperature, then with FITC or PerCP-labeled anti-CD8 Ab (BD Pharmingen) for 15 min at 4°C, followed by two washes in FACS buffer. Samples were acquired on FACScan or FACSCalibur flow cytometers (BD Biosciences). Data were analyzed using CellQuest software (BD Biosciences).

Adoptive cell transfer of HY-specific CD8 T cells

Female CBA were immunized (i.v.) three times at monthly intervals with 5×10^6 male CBA splenocytes. PBMCs were stained using HYD^k and HYK^k tetramers and the splenocytes from mice with significant tetramer staining CD8 T cell populations were pooled and used for enrichment. CD8 T cells were first purified by depletion of $B220^+$ and $CD4^+$ T cells using Dynabeads (Dynal). Cells were then stained with allophycocyanin-conjugated HYD^k and HYK^k tetramer then enriched using anti-allophycocyanin microbeads (Miltenyi Biotec). A total of 10^5 cells were adoptively transferred (i.v.) to each recipient.

Skin grafting

Tail skin was grafted onto the lateral thorax following the method of Billingham and Medawar. After removal of the plaster casts, grafts were observed daily and scored as rejected when <10% viable tissue remained.

Results

Defective $CD4^+$ T help compromises CD8 T cell expansion

$H2^k$ female mice rarely reject male skin despite the potential to make HY-specific CD8 and CD4 T cell responses directed at the immunodominant class I epitope, HYD^k, and the HYE^k class II epitope, respectively. To explore the mechanism(s) limiting the response, we first characterized the immunodominant HYD^k-specific CD8 T response (19) in the $H2^k$ strains CBA and B10.BR. Females were immunized with male spleen cells and peripheral blood analyzed using HYD^k*Smcy* tetramer. None of the $H2^k$ recipients gave a detectable primary HYD^k response and a secondary response was seen in just one of five CBA recipients (Fig. 1, *A–C*). By contrast, (C57BL/6 × CBA)F_1 $H2^{bxk}$ responders gave robust primary HYD^k*Smcy* responses (Fig. 1, *A* and *D*). These data show that effective CD4 T cell help leading to efficient expansion of

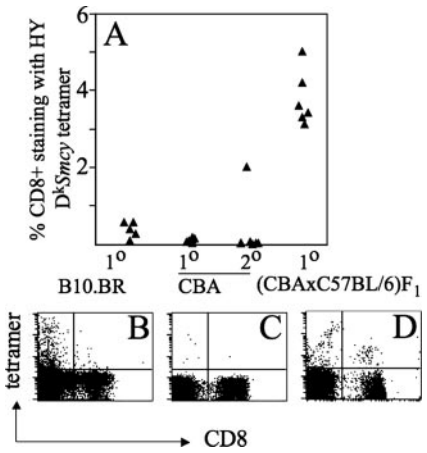

FIGURE 1. Lack of D^kSmcy response in $H2^k$ strains. *A*, $H2^k$ strains, CBA, and B10.BR but not (CBA × C57BL/6)F_1 fail to expand HYD^k-specific CD8 T cells in response to 1^0 and 2^0 immunization with male spleen cells. PBL were stained with HYD^k tetramer. Representative tetramer stains of PBL from CBA (*B*), B10.BR (*C*), and (CBA × C57BL/6)F_1 (*D*).

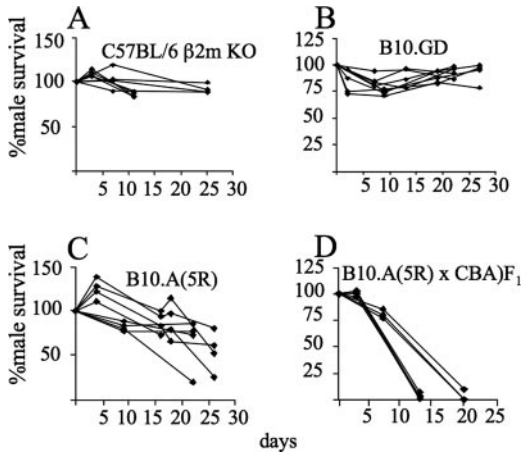

FIGURE 3. Survival of male hemopoietic cell grafts in females. Recipients were administered (i.v.) with syngeneic male and female splenocytes (10^7 each) differentially labeled with CFSE. Labeled populations were quantified in serial blood samples and relative survival of male cells plotted. *A*, β2m KO; *B*, B10.GD; *C*, and B10.A(5R) and *D*, (B10.A(5R) × CBA)F_1.

naive HYD^k CD8 T cells is provided by the HYA^b but not the HYE^k helper response. The robust CD8 HY responses of $H2^b$ strains and (C57BL/6 × CBA)F_1 mice (19, 24) confirm that HYA^b confers dominant responsiveness. The nonresponsiveness of the B10.BR strain which has a genetic background closely related to the robust HY responder strain C57BL/10 makes it unlikely that non-MHC linked genes play a significant role in modulating the CD8 HY response.

To assess whether ineffective help delivered by HYE^k reflects the size of the responding CD4 T cell pool, the ex vivo IFN-γ ELISPOT assay was used to estimate the frequencies of HYE^k- and HYA^b-specific T cells from $H2^{bxk}$ (C57BL/6 × CBA)F_1 females which had rejected male skin 160 days earlier. Fig. 2 shows that HYE^k- and HYA^b-specific T cells have similar frequencies, at least in F_1 hybrids, suggesting the failure of the HYE^k response to provide effective help is unlikely to be attributable to lack of expansion and IFN-γ production.

Variable responsiveness of $H2^k$ strains to male hemopoietic cell grafts

Development of HYD^k-specific CD8 T cells in a proportion of $H2^k$ females receiving two immunizations (Fig. 1*A*) suggested considerable individual variability. To explore this, we used a more sensitive readout of HY immunity by following the specific elimination of adoptively transferred male spleen cells (23). We first established that rejection of male hemopoietic cells, as for skin, requires participation of both CD4 and CD8 T cells (10, 11). β_2m KO mice have a normal CD4 T compartment but have severely compromised CD8 T cell immunity. Conversely, B10.GD (K^b, A^d, D^b) share MHC class I alleles with B6 but selectively lacks HY-specific help (16). Fig. 3, *A* and *B*, shows that male hemopoietic cells survive throughout the time course of this experiment in female β_2m KO and B10.GD recipients while C57BL/6 females completely reject male hemopoietic cell grafts (Ref. 23 and see Fig. 5) confirming neither T cell subset alone can eliminate male hemopoietic cells.

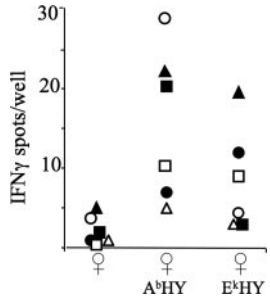

FIGURE 2. A^bDby and E^kDby CD4 T cell responses are quantitatively similar. Estimation of E^k- and A^b-restricted HY-specific CD4 T cells by IFN-γ ELISPOT assay. Six (C57BL/6 × CBA)F_1 female recipients (□, ○, △, ●, ■, and ▲) were immunized with male skin grafts and analyzed 120 days later. Numbers of responding cells are the averages of three ELISPOT wells each containing 2×10^5 spleen cells. The values are for control female spleen cells and female spleen cells coated with 10 μM HYA^k and HYE^k peptides.

We next determined whether CD8 T cells recognizing the $H2^k$ class I HY epitopes are competent to reject male hemopoietic cells. B10.A(5R) (K^b, A^b, D^d) and (B10.A(5R) × CBA)F_1 strains both present the dominant HYA^b helper epitope, but the $H2^k$-restricted HY-specific epitopes (HYD^kSmcy and HYK^kSmcy) are presented only by the F_1 hybrid. The response of these mice to male cells is shown in Fig. 3, *C* and *D*. Male F_1 spleen cells were rapidly and completely eliminated by syngeneic F_1 female recipients while B10.A(5R) females all partially rejected syngeneic male cells with slower kinetics suggesting the class I alleles K^b and/or D^d can present (currently undefined) immunogenic HY peptide(s) to CD8 T cells. These data show that like skin grafts, rejection of male hemopoietic cells is dependent on both CD4 and CD8 T cell responses and that in the context of HYA^b-mediated CD4 help, $H2^k$-restricted CD8 T cells are competent to mediate hemopoietic cell rejection.

Unlike the consistent rejection of male cells by B6 and (B10.A(5R) × CBA)F_1 females, the response of individual CBA and B10.BR female recipients was variable. Rejection of male spleen cells was seen in three of eight and one of seven CBA (Fig. 4, *A* and *C*) and four of seven B10.BR recipients (Fig. 4*B*) while the remainder of each group were nonresponsive. 2^0 and 3^0 immunization provoked rejection responses in a further three CBA recipients while others remained nonresponsive (Fig. 4, *D* and *E*). The three most responsive mice received a fourth immunization with male spleen cells and their HY-specific CD8 responses were analyzed 2 wk later using HYD^kSmcy and HYK^kSmcy tetramers

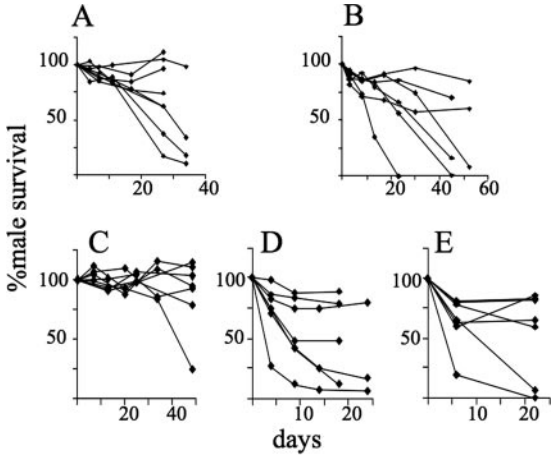

FIGURE 4. Immunity to male hemopoietic cell grafts in $H2^k$ strains. Recipients were administered (i.v.) with male and female splenocytes (10^7 each) differentially labeled with CFSE. Labeled populations were quantified in serial blood samples and relative survival of male cells plotted. *A*, CBA; *B*, B10.BR; *C–E*, primary, secondary, and tertiary immunization of CBA.

which detected HY-specific CD8 T cells only in the initial responder (data not shown). These data show that functional in vivo immunity to male spleen grafts develops only in a proportion of multiply immunized $H2^k$ recipients and detectable expansion of HY-specific CD8 T cells occurs only in a proportion of responders.

$H2^k$-restricted HY-specific CD4 and CD8 T cells are susceptible to regulation by CD4 $CD25^+$ Tregs

Nonresponsiveness to male cells in a proportion of $H2^k$ females despite multiple immunization is consistent with the establishment of dominant regulation which actively maintains in vivo nonresponsiveness. Tregs controlling HY immunity in $H2^k$ strains could derive from three sources: 1) pre-existing HYE^k-specific Treg; 2) HYE^k-specific Tregs which differentiate from naive HYE^k CD4 T cells following Ag encounter; 3) non-HYE^k-specific Tregs. Although it is difficult to distinguish between these possibilities, we have investigated the susceptibility of HYA^b- and HYE^k-specific CD4 T cells to regulation. Rag KO TCR-transgenic mice expressing the A1 (HYE^k) TCR or Marilyn (HYA^b) TCRs contain very few natural $CD25^+$CD4 Tregs (data not shown) and both reject male skin grafts (21, 25). In contrast, Rag-sufficient A1 and Marilyn TCR-transgenic females contain $CD25^+$ CD4 Treg populations arising through rearrangement of endogenous TCR chains directing differentiation into the $CD25^+$ Treg lineage. Fig. 5, *A* and *B*, shows the characteristics of the peripheral $CD25^-$ and $CD25^+$CD4 populations in Rag sufficient A1 and Marilyn strains. First, the ratio of $CD25^-$ to $CD25^+$ CD4 T cells is around 14 times higher in A1 (A1: 99.7:0.3 = 332:1; Marilyn 96:4 = 24:1, respectively, Fig. 5*A*). Second, most Marilyn $CD25^+$CD4 T cells express the transgenic Vβ6 chain (>96% positive, Fig. 5*B*) and can be activated by the cognate peptide demonstrating functional expression of the TCR (data not shown). Similarly, the majority (~75%) of A1 $CD25^+$ CD4 Treg express the transgenic Vβ8.2 chain. Despite Rag-sufficient A1 having a much higher effector-Treg ratio within the regulatory compartment, HY-specific regulation in vivo is a feature of Rag^+ Al but not Rag^+ Marilyn. This is evident as the presence of the large Marilyn $CD25^+$ CD4 Treg population does not influence the rejection of male skin (data not shown) while Rag-sufficient A1 females do not reject skin grafts unless the $CD25^+$CD4 Treg population is depleted (14).

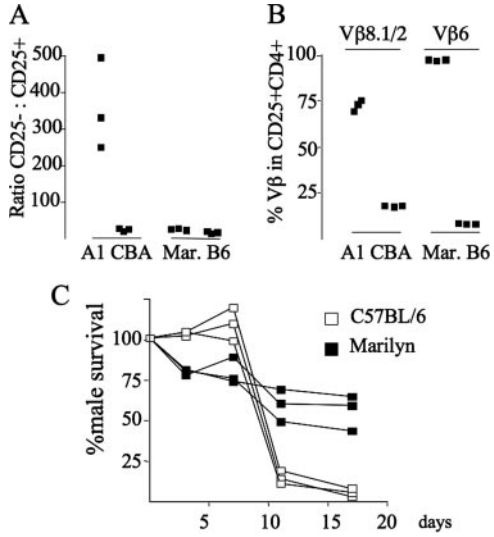

FIGURE 5. Characterization of natural Treg cells and immunity in Rag^+ TCR transgenic A1 and Marilyn strains. *A*, Ratio of $CD25^-$:$CD25^+$ CD4 T cells in Rag^+-transgenic A1 and Marilyn and control CBA and C57BL/6 mice ($n = 3$ for each strain). *B*, Percentage of transgenic TCR Vβ expression on $CD25^+$ CD4 T cells in Rag^+-transgenic A1 and Marilyn and control CBA and C57BL/6 mice ($n = 3$ for each strain). *C*, Survival of male hemopoietic cell grafts in Rag^+ TCR-transgenic Marilyn and B6 recipients. Female recipients were administered (i.v.) with male and female splenocytes (10^7 each) differentially labeled with CFSE. Labeled populations were quantified in serial blood samples and relative survival of male cells plotted.

To further assess HY-specific regulation in A1 and Marilyn Rag-sufficient mice, responses to male hemopoietic T cells were compared. Fig. 5*C* shows that female Rag^+ Marilyn females all rapidly reject ~50% of the adoptively transferred male spleen cells corresponding to the MHC class II^+ subset (data not shown). The effector HY-specific $CD4^+$ T cell population is thus not subject to functional regulation by the $CD25^+$CD4 Treg population. A very different outcome was seen in A1 recipients in which clear primary immunity to male cells was seen in only two of eight recipients (Fig. 6*A*); these two recipients also responded robustly to subsequent 2^o and 3^o inoculations of male cells (Fig. 6, *B* and *C*). The remaining A1 recipients were tolerant to 1^o, 2^o, and 3^o inocula of

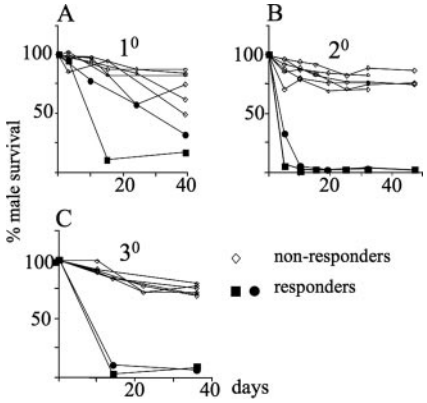

FIGURE 6. Survival of male hemopoietic cell grafts in Rag^+ TCR-transgenic A1 recipients. Female A1 recipients were administered (i.v.) with male and female splenocytes (10^7 each) differentially labeled with CFSE. Labeled populations were quantified in serial blood samples and relative survival of male cells plotted. *A–C*, Primary, secondary, and tertiary immunization, respectively.

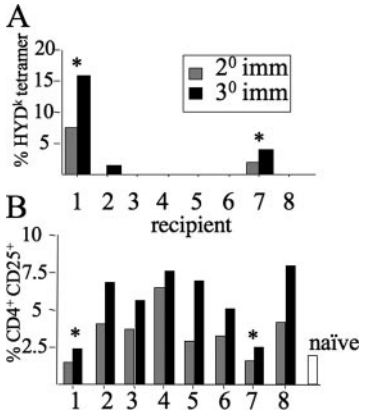

FIGURE 7. HY-specific CD8 T and CD25$^+$ CD4 T cell populations in responsive and nonresponsive A1 recipients. Al recipients were analyzed after the second and third immunizations. ∗, The two responders (1, 7). Percentage of HYD^k tetramer-specific CD8 T cells (*A*) and CD25$^+$ CD4 T cells (*B*) are shown. The percentage of CD25$^+$CD4 T cells in a control naive A1 Rag$^+$ mouse is indicated.

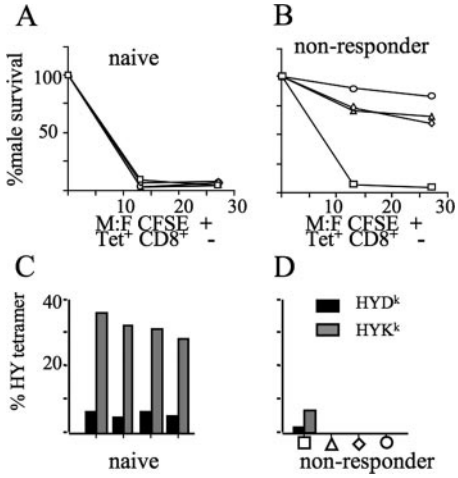

FIGURE 8. Tolerant A1 recipients suppress expansion and in vivo cytotoxicity of adoptively transferred HY-specific CD8 T cells. Naive (*A*) or nonresponder (*B*) A1 recipients were administered (i.v.) with male and female splenocytes (10^7 each) differentially labeled with CFSE and 10^5 HYD^k/K^k-specific CD8 T cells as indicated below each panel. Labeled populations were quantified in serial blood samples and relative survival of male cells plotted. *C* and *D*, Expansion of the transferred HY-specific CD8 T cells was measured by tetramer analysis of PBL.

male cells. The behavior of the Rag$^+$ A1 and Marilyn TCR-transgenic mice thus recapitulate the responses of nontransgenic H2^k and H2^b strains to male spleen cells and support the notion that the HYE^k response is particularly susceptible to regulation by natural CD25$^+$ CD4 T cells. In contrast, the naive CD4 T cell compartment in Rag$^+$ Marilyn is less susceptible to regulation despite the repertoire having a much higher proportion of HY-specific CD25$^+$ CD4 Tregs.

The state of tolerance persisting in most A1 recipients following inoculation of male spleen cells was investigated by analysis of peripheral blood for HY-specific CD8 T cells and CD25$^+$ CD4 T cells. Both responders but only one of six tolerant A1 mice had detectable HYD^k*Smcy*-specific CD8 T cell populations (Fig. 7*A*). Conversely, the CD25$^+$CD4 T cell populations gave the reverse picture remaining at the level of the naive controls in both responders while the nonresponders all had larger proportions of CD25$^+$CD4 T cells (Fig. 7*B*).

To provide direct evidence of enhanced Treg function in nonresponsive A1 recipients, 10^5 HYD^k/K^k*Smcy* tetramer-enriched CD8 T cells were adoptively transferred together with CBA 1:1 male/female spleen cell populations differentially labeled with CFSE into nonresponding or naive A1 recipients. In all the naive recipients, the transferred HY-specific CD8 T cells expanded and rapidly eliminated the cotransferred CFSE-labeled male spleen cells (Fig. 8, *A* and *C*). In contrast, the male cells were rejected by only one of four nonresponders. A limited expansion of the transferred CD8 T cells was also seen only in this nonresponder (Fig. 8, *B* and *D*). A further two nonresponders received only CFSE-labeled male and female spleen cells and were again tolerant of the male cells (data not shown). Overall, these data demonstrate that dominant regulation is operating in A1 but not Marilyn TCR-transgenic Rag-sufficient mice. As the larger CD25$^+$ CD4 Treg population of Marilyn would be expected to be more potent than the A1 Treg population, this result supports the proposition that A1 CD4 T cells are more susceptible to in vivo regulation than Marilyn CD4 T cells.

*Effector function of immunodominant HYD^k*Smcy*-specific CD8 T cells*

Although the lack of effective CD4 T cell help deriving from the HYE^k response and the consequent failure of CD8 effector T cells to expand may be sufficient to explain poor rejection of male skin grafts by H2^k females, we further examined the in vivo effector potential of the immunodominant HYD^k-specific CD8 effector cell population in the context of effective HYA^b-mediated help. (B10(A)5R × CBA)F$_1$ females were used as recipients of primary male skin cells grafts. In this F$_1$ hybrid strain, H2^k-restricted HY-specific CD8 T cells receive functional help from HYA^b-specific CD4 T cells (Table I). Unlike primary male hemopoietic cell grafts which were rapidly rejected (Fig. 3), primary skin grafts were accepted by around one-third of recipients (Fig. 9*A*). The lack of skin graft rejection by H2^k strains is thus not entirely explained by the lack of effective CD4 T cell help because correction of this deficit

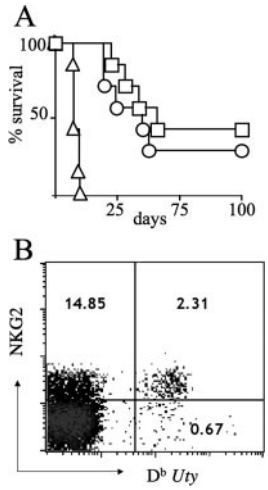

FIGURE 9. In vivo function of HYD^k/K^k CD8 T cells. *A*, Naive (two experiments, ○ and □) and male spleen cell primed (△) (B10.A(5R) × CBA)F$_1$ female recipients were grafted with male skin. Graft survival is plotted. *B*, CD94/NKG2 expression on HY-specific CD8 T cells. PBL from an immunized C57BL/6 mouse was stained with HYD^b*Uty* tetramer and anti-CD94. A high proportion of the HY tetramer staining but not the nonstaining CD8 T cells express NKG2/CD94. The staining pattern is representative of eight mice analyzed.

does not facilitate robust rejection of skin. One potential explanation of these data is that the hemopoietic cell grafts are directly removed by the effector function of the HYA^b helper CD4 T cells. This possibility is excluded because male hemopoietic cells are retained in β_2m-deficient mice which have a $H2A^b$ selected CD4 T cell repertoire but lack CD8 T cells (Fig. 3).

To assess whether the relative resistance of male skin grafts to rejection by $H2^k$-restricted CD8 T cells is overcome when the frequency of effector cells is increased, we used immunized (B10(A)5R × CBA)F_1 females and C6 TCR-transgenic (26) recipients expressing a HYK^k-specific TCR on the majority of their CD8 T cells. Rejection of male skin in both groups was complete, demonstrating the low frequency of effector CD8 T cells to be a factor in the relatively weak rejection response mediated by $H2^k$-restricted CD8 T cells (Fig. 9*A* and data not shown).

CD94/NKG2 interaction negatively regulates the HY-specific CD8 T cell response

The observation that the $H2^k$-restricted HY CD8 T cell response, while effective at eliminating hemopoietic cells, failed to reject male skin grafts in a proportion of recipients suggests additional level(s) of regulation may control immunity. One pathway which can negatively regulate CD8 T cell effector function is mediated by CD94/NKG2A following engagement with the nonclassical class I molecule Qa-1^b (27). The inhibitory CD94/NKG2A complex but not the activating CD94/NKG2B/C complexes are expressed by many activated CD8 T cells (28) and can exert a strong negative influence on in vivo T cell responses to some viruses (29–31) but not others (32) and can ameliorate GvH disease (33). In common with other CD8 T cell responses, CD94/NKG2 is up-regulated on HY-specific CD8 T cells (Fig. 9*B*).

Inhibitory signaling by CD94/NKG2A is dependent on presentation of the Qa-1 nonclassical class I MHC molecule in combination with the Qdm peptide encoded in the leader sequence of H2-D MHC class I alleles (27). $H2D^b$ knockout mice do not encode the Qdm peptide, due to removal of the H2-D^b leader sequence thus preventing CD94/NKG2A from engaging with Qa1. In the absence of this inhibitory pathway, the effector function of otherwise weak HY-specific CD8 T cell responses might be enhanced. In particular, the potential HYK^b-specific response observed in B10.A(5R) might be amplified. This would be apparent in the H-$2D^b$ knockout strain as H2-K^b is the only class I allele present.

To address this, H2-D^bKO, H2-K^bKO, H2-K^bD^b double KO, and wild-type (WT) C57BL/6 recipients were grafted with male and female skin. As expected from the analysis of β_2m KO mice, the double KO, which cannot make HY-specific CD8 responses, accepted male skin (Fig. 10*D*). Also, as expected, H2-K^bKO females rejected male skin (Fig. 10*B*) because the D^b allele restricts the defined functional *Smcy* and *Uty* epitopes (Table I). The D^bKO also rejected male skin with a similar tempo to the K^bKO and WT B6 groups, a result consistent with an effector HYK^b-restricted CD8 T cell response, when relieved of inhibitory signaling through the Qa1/CD94 pathway, can elicit skin graft rejection.

To rule out the possibility that the enhanced HYK^b-restricted response was due to an altered CD8 T cell repertoire selected in the absence of H2-D^b, we used (B10.A(5R) × B6)F_1 females as recipients of both B10.A(5R) and $B6D^b$ KO skin grafts. Both grafts express $H2K^b$ but only the B10.A(5R) graft will express the Qdm peptide (encoded in the H2-D^d leader sequence) allowing engagement of CD94/NKG2. Again, the D^bKO male grafts were rejected but the male B10.A(5R) male grafts were not (Fig. 10, *E* and *F*). These data confirm that, in the absence of inhibitory signaling through the CD94/NKG2 pathway, the functional activity of CD8 T cell responses directed at transplantation Ag expressed on skin can be markedly enhanced.

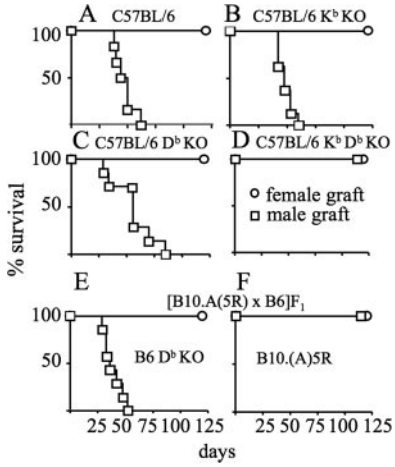

FIGURE 10. HY-specific $H2K^b$-restricted CD8 T cell reject skin grafts in the absence of NKG2/CD94 signaling. Survival of male (□) and female (○) skin grafts on C57BL/6 (*A*), C57BL/6 K^b KO (*B*), C57BL/6 D^b KO (*C*), C57BL/6 K^bD^b KO (*D*). Survival of male (□) and female (○) C57BL/6 D^b KO (*E*), and B10.A(5R) (*F*) skin grafts on female (B10.A(5R) × B6)F_1 recipients.

Discussion

Defective CD4 help

Genetic and molecular characterization of autosomal minor histocompatibility loci has shown the consistent presence of linked genes encoding epitopes presented by MHC class I and II molecules. Because these loci have been genetically isolated by selective breeding for their ability to mediate skin graft rejection, a necessity for competent CD4 and CD8 T cell responses was inferred and subsequently confirmed by the genetic dissection of individual minor histocompatibility loci (34). Interestingly, rejection of skin but not heart grafts expressing multiple minor Ags can be mediated by either T cell subset (8). Conversely, the Y chromosome, a genetic region which has not been experimentally selected for expression of minor histocompatibility Ags, encodes unique peptides presented by MHC class I and II and able to stimulate robust rejection of male skin graft in the context $H2^b$ but not other haplotypes. Our analysis of immunity to male tissue associated with other MHC haplotypes has revealed mechanisms which naturally regulate T cell responses to minor histocompatibility Ags. The mechanisms uncovered would be difficult to identify where functional outcome has been predetermined, such as the autosomal minor histocompatibility Ags.

In various experimental systems, secondary and memory responses can be severely affected while primary CD8 T cell responses are relatively unaffected in the absence of CD4 T cell help (12, 35). In contrast, the primary HYD^k CD8 T cell response appears to have a strict requirement for CD4 help mediated by HYA^b-specific CD4 T cells. The HYD^b*Uty* response is similarly dependent on HYA^b CD4 T cells (24). Helper CD4 T cells recognizing a tetanus peptide delivered via DNA vaccination are also able to provide effective help for the HYD^b*Uty* response (36). Actively maintained tolerance, as seen in a proportion of WT and TCR-transgenic $H2^k$ female recipients described here, may contribute to the lack of primary CD8 T cell responses in the presence of weak CD4 T cell help. CD4 T cell-mediated regulation is not however required for the suppression of HY CD8 responses because MHC class II-deficient C57BL/6 also fail to give primary HY CD8 responses (37).

It is not clear why primary CD8 responses to minor histocompatibility Ags can be more dependent on CD4 help than primary CD8 responses to other epitopes. Transfer ex vivo of spleen cells may provide very limited "danger" signals in comparison with other immunizing cell populations. Reports showing CD4-independent CD8 T cell responses have generally used immunization with cell populations which may not reflect normal physiology, for example MHC class I-deficient tumor cells (12) or high frequencies of adoptively transferred TCR-transgenic CD8 T cells (35). MHC class I deficiency is the prototypic trigger for NK cell activation and abnormal cell physiology can be detected via activating receptors on unconventional T cells whose ligands are induced by cell transformation, infection, and stress (38). The lack of MHC class I, transformed phenotype, and stress induced by extended in vitro culture of the inoculated cells may evoke significant activation of the innate immune system and cytokine production. Relevant to this possibility, production of IL-12 by activated NK cells (39) and production of type I IFNs by activated dendritic cells viral following viral infection (40) can provide help for CD8 T cell expansion in the absence of CD4 T cells.

The HYE^k response does however provide limited help as a proportion of $H2^k$ recipients reject male hemopoietic cells and develop HY-specific CD8 T cell populations after multiple immunization. IFN-γ ELISPOT analysis showed quantitatively similar HYE^k and HYA^b responses in F_1 mice suggesting a functional deficiency of the HYE^k population. However, the caveat that the HYA^b response may have aided the HYE^k response through IL-2 production or other means cannot be discounted. Despite the limited help provided via HYE^k recognition, a fraction of recipients remain nonresponsive despite multiple immunization with male hemopoietic cells. These data suggest that immunity to HY in $H2^k$ strains is delicately balanced. To gain insight into the mechanism(s) underlying the dichotic response, we analyzed Rag-sufficient transgenic mice expressing HYE^k- or HYA^b-specific TCRs which showed that, in comparison with the HYA^b response, the HYE^k response was extremely susceptible to regulation by $CD25^+$ CD4 T cells. Despite the much higher proportion of HY-specific regulatory CD4 $CD25^+$ T cells present in the Marilyn repertoire, effector CD4 cells rapidly reject male cells. The susceptibility of the HYE^k response to regulation in the Rag^+ A1 TCR-transgenic recipients led to dichotic responses to male hemopoietic cells with a proportion of recipients remaining nonresponsive despite multiple immunization similar to the responses of WT $H2^k$ recipients. Nonresponsiveness was associated with active regulation as adoptively transferred, primed HY-specific CD8 T cells were unable to expand. It is not clear why Ag-specific, naive CD4 T cells have very different susceptibilities to regulation following exposure to Ag. Susceptibility may be cell intrinsic or may reflect the balance of the activating and regulating signals being received. The differential behavior of the two transgenic strains provides an attractive model system to dissect factors regulating the interplay between naive and regulatory CD4 T cells. These data strongly suggest nonresponsiveness to HY in $H2^k$ strains may also involve active regulation by natural or induced Treg populations. These observations may also have relevance to immunity in immunosuppressed individuals where the balance of effector and regulatory function may be perturbed.

Effector function

Perreault et al. (41) have shown that adoptively transferred CD8 T cells specific for a single, dominant minor histocompatibility mismatch eliminated leukemic cells without causing GvH disease despite broad expression of the Ag. Using skin and hemopoietic cell grafts as targets for the $H2^k$ CD8 response, we also find hemopoietic cells to be more susceptible to elimination although a proportion of skin grafts were rejected. For individual minor histocompatibility Ags, relative tissue susceptibility is likely to differ and be controlled by multiple factors including epitope density and properties of the T cell repertoire. For example, although the $H2^k$-restricted CD8 T cell response to HY is immunodominant over the $H2^b$ response (19) and both reject hemopoietic cell grafts, $H2^b$-restricted CD8 T cells are more effective at eliciting skin graft rejection. The most attractive targets for promoting the graft-vs-leukemia effect without GvH disease are, however, those minor histocompatibility Ags whose expression is restricted to hemopoietic lineages (42).

CD8 T cell effector function can be down-modulated by negative signaling following CD94/NKG2A engagement with the Qa1/Qdm. This pathway has been shown to critically affect the outcome of polyoma virus and HSV (29–31) but not lymphocytic choriomeningitis virus (32) infection through limiting CD8 T cell expansion and function. CD94/NKG2 is expressed on most NK cells during the neonatal period and a smaller population in the adult (43). Its ligand is composed of the nonclassical class I molecule Qa1 and the Qdm peptide derived from the leader sequence of *H2-D* locus class I molecules (27). NK cells monitor Qa1/Qdm as a pan-haplotype indicator of MHC class I expression. Following activation, many CD8 T cells also express CD94/NKG2 and other NK receptors. We show this includes CD8 T cells responding to the HYD^b*Uty* complex. As well as direct inhibitory signaling following engagement of Qa1/Qdm, a population of regulatory CD8 T cells recognize Qa1 complexed with TCR-derived peptides (44). To focus on the impact of the direct CD94/NKG2 pathway on modulating CD8 immunity, we used H2-D^b-deficient mice which do not produce the Qdm peptide thus disabling inhibitory signaling via the CD94/NKG2 pathway. In this strain, the Qa1 molecule is expressed and will associate normally with other peptides. As H2-D^b presents the two defined CD8 T cell HY epitopes, use of H2-D^b-deficient mice may reveal normally weak responses which are enhanced in the absence of Qa1/Qdm expression. Strikingly, we found female H2-D^b KO mice, but not female H2-D^b, -K^b double KO mice reject syngeneic male skin. Further, by using $(B10.A(5R) \times B6)F_1$ females as recipients of male B10.A(5R) and male H2-D^b KO skin grafts, the importance of the Qdm peptide was confirmed. The role of this pathway in limiting GvH disease (33) is consistent with its impact on immunity to minor Ags expressed by skin grafts. The dramatic effect of the CD94/NKG2 pathway on modulating skin graft rejection demonstrates its potential importance in organ transplantation and identifies a potential target for down-regulating GvH while maintaining immunity to leukemic cells.

Acknowledgments

We thank Dr. Olivier Lantz for critical reading of the manuscript and Hamlata Dewchand for skin grafting.

Disclosures

The authors have no financial conflict of interest.

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