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T-Cell Activation, Proliferation, and Memory After Cardiac Transplantation *In Vivo*

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Objective

To study the response of alloantigen ($H2K^b$)-specific T cells to a $H2^{b+}$ cardiac allograft *in vivo*.

Summary Background Data

The response of T cells to alloantigen has been well characterized *in vitro* but has proved more difficult to assess *in vivo*. The aim of these experiments was to develop a model of T-cell-mediated rejection where the response of T cells after transplantation of a cardiac allograft could be followed *in vivo*.

Methods

Purified $CD8^+$ T cells from $H2K^b$-specific TCR transgenic mice (BM3; $H2^k$) were adoptively transferred into thymectomized, T-cell-depleted CBA/Ca ($H2^k$) mice. These mice were then transplanted with a $H2K^{b+}$ cardiac allograft. Using four-color flow cytometry, the proliferative response, modulation of activation markers, and potential cytokine production of the $H2K^b$-specific T cells was assessed after transplantation.

Results

Consistent rejection of $H2K^{b+}$ cardiac allografts required the transfer of at least 6×10^6 $CD8^+$ $H2K^b$-specific T cells. Short-term analyses revealed that the transgenic-TCR^+/$CD8^+$ T cells proliferated and became activated after transplantation of an $H2K^{b+}$ cardiac allograft. Fifty days after transplantation, the transgenic-TCR^+/$CD8^+$ T cells remained readily detectable, bore a predominantly memory phenotype ($CD44^{hi}$), and rapidly produced interleukin 2 and interferon-gamma on *in vitro* restimulation.

Conclusions

These data show that the activation of alloantigen-specific T cells can be followed *in vivo* in short-term and long-term experiments, thereby providing a unique opportunity to study the mechanisms by which T cells respond to allografts *in vivo*.

T cells have been identified as the cells responsible for the initiation of the destructive immune response mounted against allogeneic tissue grafts.[1–4] In general, T cells can be divided into two major subsets according to whether they express CD4 (for MHC class II-restricted responses) or CD8 (for MHC class I-restricted responses).[5]

The immune response targeting MHC-mismatched allografts has been demonstrated, both *in vitro*[6] and *in vivo*,[7] to result predominantly from the recognition of intact allo-MHC molecules (+ peptide) present on either the allograft itself or on donor passenger leukocytes. This form of recognition has been termed the direct pathway.[7] A second pathway of allorecognition, termed the indirect pathway, whereby peptides derived from allogeneic MHC molecules are presented by recipient antigen-presenting cells, also participates.[8]

After the appropriate recognition of alloantigen, T cells direct the immune system's numerous effector mechanisms that mediate graft rejection.[9] Both $CD4^+$ and $CD8^+$ T cells contribute to allograft rejection, but controversy remains as to which cell is more important. It seems likely, however, that both $CD4^+$ and $CD8^+$ T-cell subsets are capable of contributing to the rejection process[3,10–12] by supplying the cytokines necessary for the generation of effector mechanisms, including cytotoxic T lymphocyte cytolysis[13,14] and delayed-type hypersensitivity.[3,10] The relative contribution of each subset of T cells appears to depend in part on the antigenic disparity between the organ donor and the recipient[15] and the strain combination used.

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T cells have been shown to proliferate, generate cytotoxic T lymphocytes, and produce a range of inflammatory cytokines on allogeneic stimulation *in vitro*. However, because of the low precursor frequency of alloantigen-specific T cells and the lack of T-cell receptor (TCR) Vβ restriction to the majority of alloantigens *in vivo*,[16] few studies have been able to investigate the response of T cells to a defined alloantigen expressed by a tissue graft *in vivo*.

To overcome these problems, we have developed a model in which $CD8^+$ T cells with a defined specificity for the MHC class I molecule $H2K^b$ were transferred into thymectomized, T-cell-depleted CBA ($H2^k$) mice. The $H2K^b$-specific T cells were purified from BM3 TCR transgenic (Tg) mice,[17,18] which have large numbers of T cells that recognize the intact $H2K^b$ molecule directly. These T cells can be identified using the anticlonotypic TCR monoclonal antibody Ti98.[19,20] The $CD8^+$ Tg-TCR^+ T cells from these mice have previously been shown to develop into cytotoxic T lymphocytes, proliferate, and produce interleukin 2 (IL-2) after stimulation with $H2K^b$ *in vitro*.[17]

In this study, the response of the $H2K^b$-specific T cells to the $H2K^b$ alloantigen expressed by a cardiac allograft was followed for 7 days after transplantation. Mice were also studied 50 days after the transplant to determine the phenotype and cytokine-producing capabilities of putative memory $H2K^b$-specific T cells.

METHODS

Animals

CBA/Ca ($H2^k$) and C57BL/10 ($H2^b$) mice were purchased from Harlan Olac Ltd. (Bicester, UK). BM3 ($H2^k$, BM3.3 TCR Tg) mice were a kind gift from Dr. A.L. Mellor (IMMG, Augusta, GA) and are now bred at the Biomedical Services Unit, John Radcliffe Hospital.[17,18] All mice were housed in the part-barrier facilities of the Biomedical Services Unit, John Radcliffe Hospital. All donor and recipient experimental mice were sex- and age-matched between 8 and 12 weeks of age at the time of the first experimental procedure.

Monoclonal Antibodies and Hybridomas

YTA3.1 and YTS169 hybridomas were a gift from Prof. H. Waldmann of the Sir William Dunn School of Pathology, Oxford University.[21] Ti98 hybridoma was a gift from Dr. A.L. Mellor.[20] All hybridomas and cell lines were grown in either RPMI 1640 or α-MEM (Gibco BRL, Life Technologies, Paisley, Scotland), supplemented with 2 mM glutamine, antibiotics, and 10% (v/v) fetal calf serum (FCS; Gibco BRL).

Anti-CD4 (YTA3.1) and anti-CD8 (YTS169) monoclonal antibodies were purified[22] and dialyzed into phosphate-buffered saline (PBS) before being used *in vivo*. Anti-CD8-APC (53-6.7), anticlonotypic TCR-biotin (Ti98), anti-CD25-PE (3C7), anti-CD44-PE (IM7), anti-CD45RB-PE (16A), anti-CD62L-PE (MEL-14), and anti-CD69-PE (A1.2F3) monoclonal antibodies were used *in vitro* for fluorescent activated cell sorter (FACS) analysis. For intracellular cytokine staining, anti-IL-2-PE (JES6-5H4), anti-interferon-γ-PE (IFN-γ; XMG1.2), anti-IL-4-PE (11B11), and anti-IL-10-PE (JES5-16E3) were used. Isotype-matched monoclonal antibodies R3-34-PE (rat IgG1) and R35-38-PE (rat IgG2b) were used as controls. All monoclonal antibodies were obtained from Pharmingen (Becton Dickinson, Oxford, UK) unless stated otherwise.

Thymectomy

CBA/Ca mice were anesthetized and placed in a supine position, and a median sternotomy to the second anterior rib was performed. The thymus was then gently excised, using forceps to remove any connective tissue. The incision was closed with a running stitch with 4-0 suture (Ethicon, Surgicon, Yorkshire, UK). This procedure was performed with a success rate of >95%.

Cell Purification and Labeling

A single cell suspension was made from spleens and mesenteric lymph nodes harvested from BM3 TCR-Tg mice. The cells were then washed in MACS buffer solution (PBS, 5 mM EDTA, and 1% FCS). The leukocytes were isolated by Ficoll separation (Sigma, St. Louis, MO). The leukocytes were incubated for 15 minutes at 4°C with anti-CD8 MACS beads (Miltenyi Biotec GmbH, Berisch Gladbach, Germany) at 100 μl of beads per 900 μl buffer per 1×10^7 cells. After incubation, the cells were loaded onto a MACS LS column on a MACS magnet and were isolated by positive selection according to the manufacturer's instructions. Typically, $CD8^+$ T cells were isolated to >95% purity. More than 95% of the $CD8^+$ T cells expressed the $H2K^b$-specific Tg-TCR.

The isolated cells were incubated for 10 minutes at 37°C with 5 μM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Leiden, The Netherlands), washed twice, and resuspended in PBS ready for intravenous injection.

Heterotopic Heart Transplantation

Vascularized heterotopic heart transplants were performed essentially as documented by Corry et al.[23] The function of the transplanted hearts was followed by abdominal palpation, grading from 4 (full cardiac activity) to 0 (cessation of activity); electrocardiogram, as described by Superina et al[24]; and laparotomy (enabling direct visualization of the transplanted heart).

Protocol

CBA/Ca mice were thymectomized (day −25) and rested for 2 weeks before being treated with depleting anti-CD4

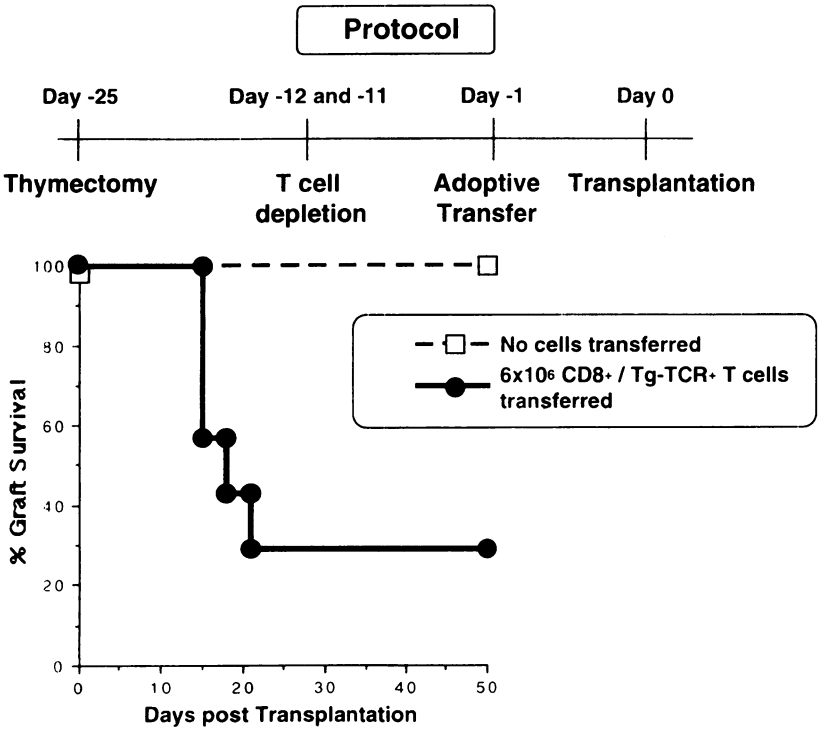

Figure 1. Immunodeficient empty CBA mice were generated by removing the thymus and depleting peripheral T cells with two doses (100 μg/dose) of depleting anti-CD4 (YTA3.1) and anti-CD8 (YTS169) monoclonal antibodies, as shown in the protocol. Ten days after the final monoclonal antibody pretreatment, 6×10^6 purified CFSE-labeled $CD8^+$ Tg-TCR^+ T cells, given intravenously, were adoptively transferred into the empty mice. The day after adoptive transfer, mice were transplanted with a B10 ($H2^{b+}$) cardiac allograft. Control empty mice also received a C57BL/10 cardiac graft but were not reconstituted with Tg-TCR^+ T cells.

(YTA3.1; 100 μg per dose) and anti-CD8 (YTS169; 100 μg per dose) monoclonal antibodies on days −12 and −11. To allow time for the depletion of the majority of T cells, the mice were rested for a further 10 days, at which time they were termed "empty" because they were severely depleted of peripheral T cells (95% T-cell depletion). The mice were then reconstituted with an intravenous injection of the CFSE-labeled purified $CD8^+$ Tg-TCR^+ (BM3) T cells (day −1). The day after adoptive transfer (*i.e.*, day 0), mice received either no cardiac allograft or an $H2K^{b+}$ (C57BL/10) cardiac allograft (see protocol in Fig. 1).

Flow Cytometric Analysis

Single cell suspensions of splenocytes and lymph node cells were prepared. 1.5×10^6 cells were aliquoted per sample and washed in PBS (Oxoid, Oxford, UK) with 0.1% (w/v) azide (Sigma) and 1% (v/v) FCS. The anti-CD8-APC and Ti98-biotin monoclonal antibodies were added to all tubes, and the samples were incubated for 30 minutes at 4°C in the dark. On completion of the incubation, the samples were washed twice with the mixture of PBS, azide, and FCS, and a streptavidin-conjugated fluorochrome was added (Pharmingen). The samples and fluorochrome were then incubated for 30 minutes at 4°C in the dark before being washed twice. Finally, the samples were incubated with a PE-labeled monoclonal antibody for detection of activation markers, followed by two washes. All samples were then fixed with 250 μl PBS with 2% (v/v) paraformaldehyde before being acquired on a FACSort and analyzed using the Cellquest software package (both Becton Dickinson).

Intracellular Cytokine Staining

Spleen cells (1×10^6/ml) were stimulated with PMA (50 ng/ml) plus ionomycin (500 ng/ml) for 4 hours at 37°C, with Brefeldin A (10 μg/ml) added for the last 2 hours. Surface staining using anti-CD8-APC and Ti98-biotin monoclonal antibodies was performed in the mixture of PBS, FCS, and azide plus Brefeldin A for 30 minutes. After two washes, streptavidin-conjugated fluorochrome was added for 15 minutes. The cells were then washed and resuspended in a mixture of PBS, Brefeldin A, and 2% (v/v) formaldehyde. The following day, cells were washed and preincubated for 10 minutes in permeabilization buffer (PBS, 1% FCS, and 0.5% saponin [Sigma]) and incubated with anti-IL2 (2.5 μg/ml), anti-IFNγ (5.0 μg/ml), anti-IL4 (5.0 μg/ml), anti-IL10 (5.0 μg/ml), or an isotype control for 30 minutes. All of the cytokine antibodies were PE conjugated. After two washes with permeabilization buffer, the cells were washed in PBS and 1% FCS without saponin to allow membrane closure. Samples were analyzed on a FACSort flow cytometer and results were analyzed using Cellquest software.

RESULTS

To study a physiologic T-cell response to a cardiac allograft *in vivo*, we required a model in which the rejection of

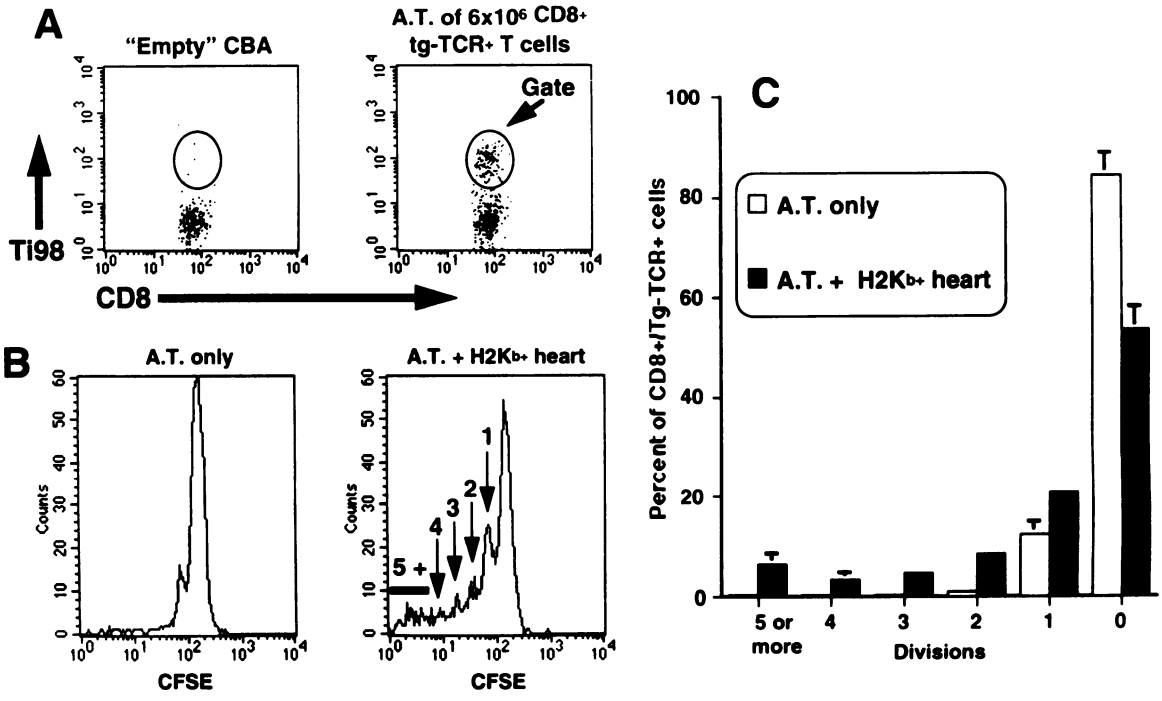

Figure 2. (A) 6 × 10^6 purified CFSE-labeled $CD8^+$ Tg-TCR^+ T cells were adoptively transferred into empty mice (AT only). Such mice were analyzed for the presence of Tg-TCR^+ T cells 1 day after transfer. Spleen leukocytes were isolated and stained for expression of CD8 and the Tg-TCR. A gate was placed around CD8-expressing cells, and the Tg-TCR expression (using the anti-Tg-TCR monoclonal antibody Ti98) on the $CD8^+$ cells was determined. For comparison, empty mice that did not receive Tg-TCR^+ T cells were also analyzed. (B) Seven days after transplantation, spleens were harvested from mice that had received $H2K^b$-specific T cells and an $H2K^{b+}$ cardiac allograft (AT + $H2K^{b+}$ heart) or untransplanted control mice (AT only). The $CD8^+$ Tg-TCR^+ T cells were analyzed using the gating procedure described in A. The proliferation of the $CD8^+$ Tg-TCR^+ T cells was assessed by analysis of the loss of CFSE fluorescence. Each successive cell division is indicated by an arrow. The bar indicates cells that have divided five or more times. (C) The bar chart represents the percentage of total $CD8^+$ Tg-TCR^+ T cells within a division peak. Results are the mean of three mice ± standard deviation (SD). The experiment was repeated three times, with similar results obtained on each occasion.

the graft could be demonstrated to be dependent on a small number of alloantigen-specific T cells. To establish this model, we generated CBA/Ca mice that were severely immunocompromised. This was achieved by removing the thymus of 8- to 12-week-old CBA/Ca mice and depleting peripheral T cells with 2 doses of 100 μg of YTA3.1 (depleting anti-CD4 monoclonal antibody) and YTS169 (depleting anti-CD8 monoclonal antibody; see protocol in Fig. 1). Such mice were depleted of 95% of peripheral T cells and were unable to reject a fully allogeneic $H2^{b+}$ cardiac allograft transplanted on day 0 (see Fig. 1; median survival time [MST] >100 days; n = 8; empty mice).

Next, we determined whether rejection of an $H2K^{b+}$ cardiac allograft by such empty mice could be reconstituted by adoptive transfer of 6 × 10^6 purified $CD8^+$ Tg-TCR^+ $H2K^b$-specific T cells before transplantation. As shown in Figure 1, adoptive transfer of 6 × 10^6 purified $CD8^+$ Tg-TCR^+ T cells (*i.e.*, $H2K^b$-specific) into empty syngeneic CBA/Ca mice the day before transplant resulted in the rejection of $H2K^{b+}$ cardiac allografts (MST 18 days, n = 7). The rejection was significantly slower than in untreated CBA/Ca mice (MST 9 days; data not shown). Empty mice adoptively transferred with 6 × 10^6 $H2K^b$-specific $CD8^+$ Tg-TCR^+ T cells were used in all subsequent experiments.

The response of $H2K^b$-specific T cells to an $H2K^{b+}$ cardiac allograft during graft rejection was investigated *in vivo*. The spleen and lymph nodes of reconstituted mice were analyzed 3, 5, and 7 days after transplantation of an $H2K^{b+}$ cardiac allograft.

CFSE is an intracellular dye that has been shown to be split equally between daughter cells after division.[25] $CD8^+$ Tg-TCR^+ T cells were labeled with CFSE before adoptive transfer. This technique enabled us to determine whether the Tg-TCR^+ T cells were stimulated to divide in response to the graft. Employing four-color flow cytometry, we gated on the $CD8^+$ Tg-TCR^+ cells from each sample, using both the anti-Tg-TCR monoclonal antibody Ti98 and an anti-CD8 monoclonal antibody (Fig. 2A). This allowed us to analyze the behavior of the $H2K^b$-specific T cells independently of other cells resident in the lymphoid organ of interest. Using this gating technique, the proliferative response of the adoptively transferred $H2K^b$-specific T cells to the cardiac allograft was determined by analysis of the CFSE profile (see Fig. 2B).

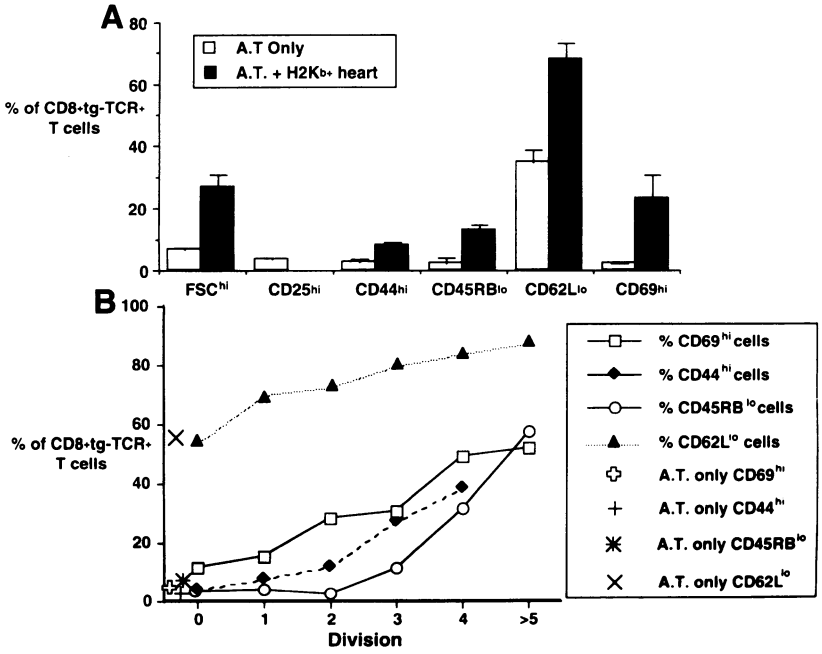

Figure 3. 6×10^6 purified CFSE-labeled $CD8^+$ Tg-TCR^+ T cells were adoptively transferred to empty mice. One group of mice received an $H2K^{b+}$ cardiac allograft (AT + $H2K^{b+}$ heart); the other group did not (AT only). The spleens of all mice were analyzed 7 days later. Using CFSE, nondividing $CD8^+$ Tg-TCR^+ T cells ($CFSE^{hi}$) from untransplanted control mice were compared with dividing cells ($CFSE^{lo}$) from mice that had received an $H2K^{b+}$ cardiac transplant. The activation status of dividing $CD8^+$ Tg-TCR^+ T cells was determined by staining for the cell surface expression of several activation markers. (A) $CD8^+$ Tg-TCR^+ T cells were stained for expression of CD25, CD44, CD45RB, CD62L, and CD69 molecules. All monoclonal antibodies were PE labeled. Isotype-matched monoclonal antibodies were used as negative controls. The size of the cells (determined by forward scatter profile) was also determined. Profiles are representative of at least eight mice studied. (B) Gating on sequential CFSE peaks (as shown in B) allowed analysis of $CD8^+$ Tg-TCR^+ T cells with a defined division history—in other words, cells were grouped according to whether they had divided once, twice, three times, and so forth after transplantation of an $H2K^b$+ heart. The percentage of cells within each cell division that had modulated expression of a given activation marker was determined.

Proliferation of $H2K^b$-specific T cells was not detectable in either the spleen or lymph nodes until 5 days after transplantation (data not shown). By 7 days after transplantation of an $H2K^{b+}$ cardiac graft, up to 50% of the $H2K^b$-specific T cells had divided at least once in both the spleen (see Figs. 2B and 2C) and lymph nodes (data not shown), as judged by loss of CFSE. However, despite T-cell proliferation, the percentage and the number of $H2K^b$-specific T cells detectable were not different from those present in untransplanted control mice. $H2K^b$-specific T cells showed only limited cell proliferation in the absence of the $H2K^{b+}$ cardiac allograft (see Figs. 2B and 2C), or when a syngeneic graft was transplanted (data not shown).

Next, we determined whether the $CD8^+$ Tg-TCR^+ T cells that were dividing 7 days after transplant of an $H2K^{b+}$ cardiac allograft exhibited an activated phenotype. The expression of CD25 (α chain of the IL-2 receptor), CD44, CD45RB, CD62L (MEL-14; L-selectin), and CD69 molecules on the dividing $H2K^b$-specific T cells was analyzed (Fig. 3). Seven days after transplantation of an $H2K^{b+}$ cardiac allograft, the dividing cells showed a two- to fourfold increase in the percentage of blasted (FSC^{hi}) cells that had upregulated CD44 and CD69 and downregulated CD45RB and CD62L compared with $H2K^b$-specific T cells isolated from control mice, suggesting that the dividing cells were activated (see Fig. 3A). Interestingly, CD25 expression was absent at all time points studied.

Using CFSE, the $H2K^b$-specific T cells were grouped according to their division history (as indicated by the CFSE peaks; see Fig. 2B) and analyzed for the expression of activation markers. We found that there was an increased number of cells that had upregulated CD44 and CD69 and downregulated CD62L expression with each successive division of $H2K^b$-specific T cells in response to the cardiac allograft (see Fig. 3B). However, CD45RB expression remained high on cells that had divided twice or less and was downregulated only on cells that had divided at least three times (see Fig. 3B).

The number, phenotype, and *in vitro* cytokine-producing capabilities of the $H2K^b$-specific T cells was then investigated 50 days after transplantation, when rejection of the graft had occurred (see Fig. 1). We found that the percentage and the number of $H2K^b$-specific T cells in the spleen (see Fig. 4A) and lymph nodes (data not shown) of mice after adoptive transfer of $H2K^b$-specific T cells and transplantation of an $H2K^{b+}$ cardiac allograft were decreased three- to fivefold compared with the numbers of $H2K^b$-specific T cells present in untransplanted controls.

Analysis of the phenotype of $H2K^b$-specific T cells in the spleen (see Fig. 4B) of mice that had been transplanted with an $H2K^{b+}$ cardiac allograft showed a profound difference in phenotype compared with those present in control mice. The $H2K^b$-specific T cells were predominantly of the $CD44^{hi}$, $CD45RB^{lo}$, and $CD62L^{lo}$ phenotype. However, the cells were not blasted and did not express CD25 (see Fig. 4B).

The ability of these putative memory T cells to produce cytokine *in vitro* when stimulated for 4 hours by ionomycin and PMA was also determined. Although fewer than 5% of the $H2K^b$-specific T cells produced either IL-2 or IFNγ after *in vitro* stimulation in untransplanted control mice (Fig. 5), a much higher percentage of $H2K^b$-specific T cells were able to produce IL-2 (26%) and IFN-γ (44%) in mice that

A

Pretreatment	% CD8+Tg-TCR+ T cells	Absolute number of CD8+Tg-TCR+ T cells
A.T. only	0.93 +/- 0	2.65×10^5 +/- 0.35×10^5
A.T. + $H2K^{b+}$ Heart	0.29 +/- 0.1	0.5×10^5 +/- 0.45×10^5

B

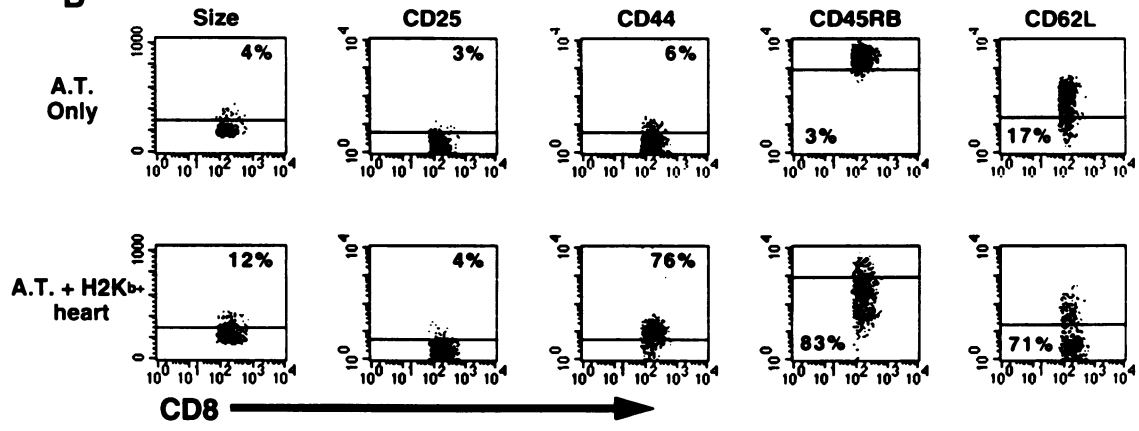

Figure 4. 6×10^6 purified CFSE-labeled $CD8^+$ Tg-TCR^+ T cells were adoptively transferred to empty mice. One group of mice received an $H2K^{b+}$ cardiac allograft (AT + $H2K^{b+}$ heart); the other group did not (AT only). The spleens of all mice were analyzed 50 days after transplantation. (A) The percentage or absolute number of $CD8^+$ Tg-TCR^+ T cells in the spleen was determined. The percentage of $CD8^+$ Tg-TCR^+ T cells was determined by CD8 and anti-Tg-TCR staining of splenocytes. Absolute numbers were calculated by multiplying the percentage of $CD8^+$ Tg-TCR^+ T cells by the number of cells in the spleen, as determined by counting under a light microscope (counting only cells that did not stain with trypan blue). The results are given as the mean ± SD; n = 3. (B) $CD8^+$ Tg-TCR^+ T cells were stained for expression of CD25, CD44, CD45RB, CD62L, and CD69 molecules. All monoclonal antibodies were PE labeled. Isotype-matched monoclonal antibodies were used as negative controls. The size of the cells (determined by forward scatter profile) was also determined. Profiles are representative of five mice studied.

had been transplanted with an $H2K^{b+}$ cardiac allograft. Interestingly, about 5% of $H2K^b$-specific T cells from these mice expressed IL-4 and/or IL-10 (see Fig. 5).

DISCUSSION

The aim of this study was to investigate the response of alloantigen-specific (anti-$H2K^b$) T cells *in vivo* after transplantation of an $H2K^{b+}$ cardiac allograft. Similar studies have been hampered by the lack of markers to identify alloantigen-specific T cells; therefore, we adopted a strategy based on that of Kearney et al.[26] The model consisted of the adoptive transfer of a limited number of purified $H2K^b$-specific $CD8^+$ T cells into syngeneic mice that had been thymectomized and depleted of T cells. The thymectomized and T-cell-depleted mice were shown to be immunocompromised and unable to reject an $H2K^{b+}$ cardiac allograft (see Fig. 1). This allowed us to determine how many $H2K^b$-specific $CD8^+$ Tg-TCR^+ T cells were required to elicit rejection of an $H2K^{b+}$ cardiac allograft and, using an anti-Tg-TCR monoclonal antibody, to follow the response of the $H2K^b$-specific T cells *in vivo* after transplantation of the graft.

6×10^6 $H2K^b$-specific $CD8^+$ Tg-TCR^+ T cells resulted in acute rejection of $H2K^{b+}$ cardiac allografts (see Fig. 1). As defined by our functional criteria for "complete" cardiac allograft rejection[24]—in other words, no electrocardiographic or detectable contractions via graft palpation—30% of the cardiac allografts failed to be rejected completely. Nevertheless, histologic analysis of these grafts revealed markedly damaged and fibrotic tissue, similar to that observed for grafts that were rejected by our functional definition (data not shown). Because the model was designed to study the response *in vivo* of $H2K^b$-specific T cells during $H2K^{b+}$ cardiac allograft rejection, we excluded mice in which the function of the graft had not completely ceased from the day 50 analysis presented here. However, the response of the $H2K^b$-specific T cells 50 days after transplant was the same in both groups of mice. Because the short-term analyses revealed little intermouse variation, suggesting that the $H2K^b$-specific $CD8^+$ Tg-TCR^+ T cells had initially responded to the cardiac allograft similarly in all mice, no mice were excluded from the short-term study.

In preliminary experiments performed to establish this model, titration of the number of $H2K^b$-specific T cells revealed that 6×10^6 cells was the minimum required for complete rejection in this model (data not shown). We suspect that adoptive transfer of 6×10^6 $CD8^+$ Tg-TCR^+ T cells is a borderline dose for the reconstitution of complete rejection by the empty mice, and although the cells

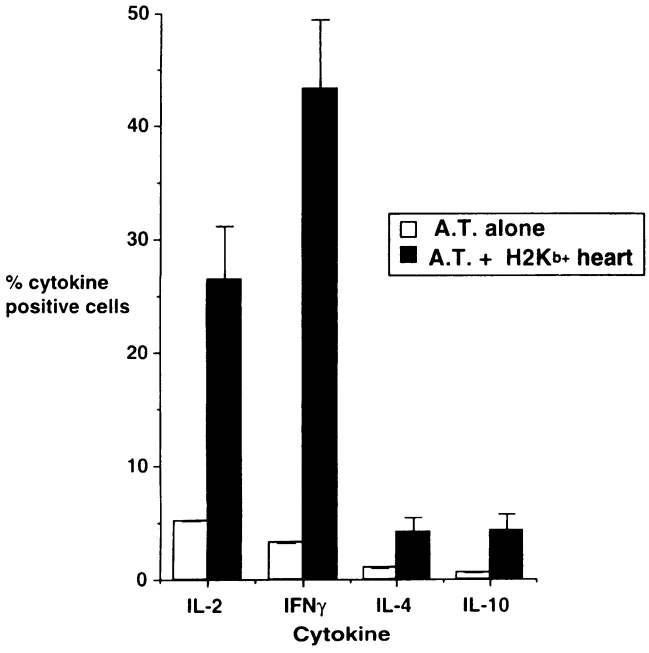

Figure 5. 6×10^6 purified CFSE-labeled $CD8^+$ Tg-TCR^+ T cells were adoptively transferred to empty mice. One group of mice received an $H2K^{b+}$ cardiac allograft (AT + $H2K^{b+}$ heart); the other group did not (AT only). The spleens of all mice were analyzed 50 days after transplantation. Splenocytes were stimulated *in vitro* with PMA and ionomycin for 4 hours. The cells were stained using anti-IL-2, anti-IFNγ, anti-IL-4, and anti-IL-10 PE-labeled monoclonal antibodies. Isotype-matched monoclonal antibodies were used as negative controls. Using four-color flow cytometry, a gate was placed around the $CD8^+$ Tg-TCR^+ T cells, allowing the evaluation of intracellular cytokine production by the $H2K^b$-specific T cells alone. Results are expressed as the mean of three mice ± SD. The experiment was repeated with similar results.

respond similarly in all mice, small intragroup variance in the number of $H2K^b$-specific T cells injected may account for the failure of some mice to reject the $H2K^{b+}$ cardiac allograft fully.

The $H2K^b$-specific T cells were shown to proliferate (see Fig. 2) and modulate expression of cell surface markers, which had been demonstrated in other systems to be associated with T-cell activation, within 7 days of transplantation of an $H2K^{b+}$ cardiac allograft (see Fig. 3). We found that the alloantigen-specific T cells triggered to proliferate after cardiac transplantation expressed increased levels of CD44 (Pgp-1) and CD69 but decreased levels of CD62L (L-selectin; Mel-14; see Fig. 3A). CD44 has been shown by others to be upregulated and CD62L downregulated on activation of $CD8^+$ T cells.[27–29] The data presented here for alloantigen-specific T cells activated *in vivo* are consistent with these findings.

We also showed that CD45RB was downregulated on activated $H2K^b$-specific $CD8^+$ T cells in the spleen after transplantation (see Fig. 3A). Changes in CD45RB expression after activation of $CD8^+$ T cells are more controversial than for the CD44 molecule. Hou and Doherty,[28] using a Sendai virus model, found that activation of $CD8^+$ T cells *in vivo* had no effect on CD45RB expression but did result in upregulation of CD44 and downregulation of CD62L. Similarly, after priming of TCR-Tg mice that recognize an influenza nucleoprotein peptide presented in the context of $H2D^b$ (F5 mice), no downregulation of CD45RB expression was observed on activated $CD8^+$ T cells.[30] In contrast, Walker et al[31] demonstrated that downregulation of CD45RB can occur *in vivo* at the peak of a $CD8^+$ T-cell response to an allogeneic tumor.

Interestingly, we found that CD45RB was downregulated only on cells that had divided at least three times. In contrast, expression of CD44, CD62L, and CD69 began to be modulated even on cells that had not yet divided (see Fig. 3B). Therefore, our data confirm that CD45RB is not coregulated with other activation markers.[30] Because CD45RB is independently regulated after activation, it is possible that with certain types of stimulus, T cells can become activated and modulate activation markers without downregulation of CD45RB. This may depend on the type and site of antigen challenge as well as the stage at which the immune response is studied. However, whether $CD45RB^{hi}$ or $CD45RB^{lo}$ $CD8^+$ T-cell subsets are functionally distinct is unclear, because a definite role of the different CD45 splice variants in T-cell activation remains to be elucidated.

Proliferation of $H2K^b$-specific T cells could not be detected until 5 days after transplantation. Interestingly, CD25 was not detected either in the spleen or lymph nodes at any of the time points studied (see Fig. 3), despite the fact that CD25 has been shown to be upregulated rapidly after $CD8^+$ T-cell activation *in vitro*.[32] Similar results have been documented *in vivo* by Chen et al[32] and Kedl and Mescher[33] after transplantation of an allogeneic cardiac and tumor graft, respectively. Both papers reported that CD25 was expressed only on cells at the graft site, suggesting that the initial sensitization of alloantigen-specific T cells occurred within the graft, or that the graft was required for full T-cell activation. Preliminary evidence using our model also suggests that $CD25^+$ T cells can be detected within the graft (unpublished observation). Although further analysis of this model is required to examine the site of sensitization after transplantation directly, our data are consistent with a role for the cardiac allograft itself in allostimulation and not simply as a target for T-cell effector mechanisms.

The phenotype and the function of $H2K^b$-specific T cells 50 days after transplantation of an $H2K^{b+}$ cardiac allograft were also determined. Surprisingly, lower numbers of $CD8^+$ Tg-TCR^+ T cells were found in the spleen (see Fig. 4B) and lymph nodes (data not shown) of mice that had rejected a cardiac allograft compared with mice that did not receive the transplant, although there was a two- to threefold increase in the number of cells with a memory phenotype (*i.e.*, $CD44^{hi}$).

These data are in stark contrast to other studies addressing T-cell memory, where the precursor frequency after primary antigenic challenge was shown to increase. However, in most of these studies a single or limited dose of

antigen was administered to prime T cells, whereas priming by a cardiac allograft may be different because the graft (and therefore the source of antigen) remains throughout the posttransplant course. Endothelial cells have been shown to be capable of stimulating T-cell responses *in vitro*.[34] It is therefore conceivable that continuous T-cell activation by the graft resulted in prolonged activation-induced cell death[35,36] and clonal exhaustion.[37,38] Alternatively, the majority of memory T cells may reside within the graft itself, with only a small proportion of cells resident in the peripheral lymphoid organs. We are currently addressing these possibilities by removing the cardiac graft at different time points after transplantation.

Increased CD44 expression has been shown to correlate with memory cell phenotype.[27,29,39] However, the modulation of CD45RB and CD62L expression by memory $CD8^+$ T cells has been demonstrated to be a less consistent marker of memory than expression of CD44. For example, memory $CD8^+$ T cells have been found to be resident in both $CD45RB^{hi}$ and $CD45RB^{lo}$ populations.[31] Similarly, a proportion of memory $CD8^+$ T cells have been shown to upregulate CD62L after an initial downregulation of the molecule at the peak of the response.[29,31,33] Therefore, $CD8^+$ memory cells can exhibit multiple phenotypes, but all reside within the $CD44^{hi}$ subset.

The $CD44^{hi}$ phenotype of $CD8^+$ Tg-TCR^+ T cells detectable in the periphery 50 days after transplantation of an $H2K^{b+}$ cardiac graft suggested that the majority had encountered the $H2K^b$ alloantigen (see Fig. 4). Consistent with the hypothesis that these cells were memory cells was the finding that large numbers of these cells were able to produce cytokine rapidly, within 4 hours of *ex vivo* stimulation with PMA and ionomycin. As expected, the majority of $CD8^+$ memory T cells had differentiated to produce IL-2 and IFNγ on restimulation, indicating a differentiation toward a Th1 phenotype rather than a Th2 phenotype.

The putative memory cells were also predominantly $CD45RB^{lo}$ and $CD62L^{lo}$, although the percentage of these cells was more variable than the percentage of $CD44^{hi}$ cells. The stability of this phenotype has not been determined, but analysis of other time points after transplantation should resolve this issue. However, 50 days after transplantation, a genuine memory T-cell pool had been generated. The significance of the fact that memory cells expressed low levels of CD45RB and CD62L remains unclear, but it is likely that the loss of these molecules would affect both activation and homing of the alloantigen-specific T cells *in vivo*.

In summary, we have developed a model in which the response of $H2K^b$-specific T cells to an $H2K^{b+}$ cardiac allograft can be followed *in vivo*. We showed that initially after transplantation, the $H2K^b$-specific T cells become activated, proliferate, and reject the $H2K^{b+}$ cardiac graft. Long-term studies showed that naïve $H2K^b$-specific T cells develop into memory cells that have a different phenotype and are functionally distinct from their naïve counterparts. This new model for studying the response of alloantigen-specific T cells *in vivo* is a powerful tool that will allow characterization of the T-cell response to other types of organ graft and provide a detailed analysis of the response of T cells under conditions that have been shown to allow transplantation tolerance to develop.

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