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# Thymic Selection by a Single MHC/Peptide Ligand Produces a Semidiverse Repertoire of CD4$^+$ T Cells

**Charles D. Surh,* Dong-Sup Lee,* Wei-ping Fung-Leung,† Lars Karlsson,† and Jonathan Sprent***

*Department of Immunology
The Scripps Research Institute
La Jolla, California 92037

†The R. W. Johnson Pharmaceutical Research Institute
3535 General Atomics Court
San Diego, California 92121

## Summary

**The influence of individual peptides in thymic selection was examined in H2-M$^-$ mice, in which positive selection is directed to a single peptide, class II–associated invariant chain peptide (CLIP) bound to H2-A$^b$. Two sensitive in vivo approaches showed that 70%–80% of CD4$^+$ T cells undergoing positive selection to CLIP+H2-A$^b$ have self-reactivity to the various peptides expressed on wild-type H2-M$^+$ antigen-presenting cells. When these self-reactive T cells were depleted, the residual CD4$^+$ cells displayed a polyclonal repertoire in terms of alloreactivity, responses to foreign protein antigens, and Vβ usage. Nevertheless, studies with two T cell receptor transgenic lines suggested that the repertoire of CD4$^+$ cells induced by CLIP was less diverse than the repertoire of CD4$^+$ cells in normal mice. Generation of a fully diverse T cell repertoire thus requires positive selection against multiple peptides.**

## Introduction

The repertoire of mature T cells is highly diverse and is formed through contact with self-peptide/major histocompatibility (MHC) complexes during early differentiation in the thymus (Bevan, 1977; Kappler et al., 1987; Sprent et al., 1988; von Boehmer, 1990; Jameson et al., 1995). Although the precise role of peptides in controlling positive selection is still unclear, there are several possibilities. First, peptide recognition by immature T cells may be highly specific, with the result that each clone of T cells is selectable by only one or a few peptides; here, T cell diversity is a direct reflection of self-peptide diversity. Second, positive selection may reflect low-affinity interaction with a limited set of self-peptides, each peptide being able to select multiple T cell specificities. Third, the main function of peptides may be to stabilize MHC molecules (which are unstable without peptides) and allow T cell receptor (TCR) recognition of MHC epitopes rather than peptides (Schumacher and Ploegh, 1994).

The role of peptides in thymic selection has been examined by testing whether specific peptides in fetal thymic organ cultures can lead to positive selection of TCR transgenic CD8$^+$ T cells bred into a class I–deficient background. Hogquist et al. (1994a, 1994b, 1995) demonstrated that positive selection of ovalbumin (OVA)–specific transgenic T cells by K$^b$ was most efficient with low-affinity antagonist peptides, whereas high-affinity agonist peptides induced either only negative selection or selection of "anergic" mature T cells that did not respond to ovalbumin antigen; no selection occurred with "null" peptides, peptides that bind to MHC but are neither antagonists nor agonists. In two other studies, positive selection of lymphocyte choriomeningitis virus (LCMV) glycoprotein-specific transgenic T cells occurred with low doses of agonist peptides, whereas high doses induced negative selection; again, weak variants of agonistic peptides induced selection whereas a null polyserine peptide failed to cause selection (Ashton-Rickardt et al., 1994; Sebzda et al., 1994). However, in contrast to the data from the anti-OVA TCR transgenic system, a moderate-agonist variant of the LCMV peptide induced positive selection of anti-LCMV TCR transgenic T cells at a wide range of concentrations without inducing negative selection at high doses (Sebzda et al., 1996). In a further study using 2C TCR transgenic mice, positive selection was mediated by a variety of unrelated peptides, including polyserine "null" peptides (Pawlowski et al., 1996). Collectively, these studies indicate that peptides are required for positive selection of CD8$^+$ T cells, but the nature of the required peptide varies depending on the system used.

More recently, the capacity of a given peptide to positively select CD4$^+$ T cells in normal mice was studied by intrathymic injection of infectious adenoviruses carrying minigene expression vectors encoding particular peptides (Nakano et al., 1997). This study found that thymic expression of peptide analogs of an agonist, with no agonist or antagonist activity, could positively select agonist-specific CD4$^+$ T cells as well as T cells specific for unrelated antigenic peptides. More important, expression of a nominal antigenic peptide was able to positively select CD4$^+$ T cells specific for the same peptide, suggesting that thymic selection cannot be explained solely by the affinity/avidity model. However, one potential problem with this system is that thymic expression of peptides induced by introducing infectious nonreplicating viral particles is a highly random event and could lead to peptide expression in only a confined region of the thymus, such as cortex but not medulla, thereby failing to induce efficient negative selection.

An alternative approach for studying the role of peptides in thymic selection is to generate mice that express only a single species of MHC/peptide complex. This has been achieved by contructing a transgenic line expressing MHC class II molecules preloaded with a peptide by covalent linkage (i.e., H2-Eα peptide attached to the H2-A$^b$ β chain [Ignatowicz et al., 1996]), and also by deleting the H2-M molecule by homologous recombination, which leads to expression of H2-A$^b$ molecules loaded only with class II–associated invariant chain peptides (CLIP) (Fung-Leung et al., 1996; Martin et al., 1996; Miyazaki et al., 1996). Surprisingly, in each situation the

presence of only a single MHC/peptide complex promoted selection of considerable numbers (25%–50% of normal) of polyclonal $CD4^+$ cells. However, in terms of primary mixed lymphocyte reactions (MLRs), a large proportion of the $CD4^+$ cells in these mice appeared to be autoreactive to syngenic H2-$A^b$ antigen-presenting cells (APCs) from wild-type mice. These findings suggest that a single MHC/peptide ligand positively selects T cells that have stronger affinity toward other peptides than toward the peptide inducing positive selection. The implication therefore is that a single MHC/peptide complex is able to positively select substantial numbers of $CD4^+$ cells but is unable to induce negative selection.

Because of the lack of negative selection in the above model, it is still unclear whether positive selection directed to a single peptide/MHC complex is able to form a diverse repertoire of T cells for *nonself* antigens. Examining this issue hinges on first removing T cells with autospecificity. In this report, we show that the proportion of $CD4^+$ T cells from H2-$M^-$ mice with reactivity to wild-type APCs is very high, at 70%–80%. Significantly, after removal of these autoreactive $CD4^+$ cells, the residual $CD4^+$ cells retained the capacity to respond to foreign antigens and were polyclonal in terms of TCR Vβ expression. The data thus support the view that contact with a restricted set of self-peptides, H2-$A^b$+CLIP, during positive selection is sufficient to generate a diverse T cell repertoire. Nevertheless, studies with TCR transgenic mice suggested that the repertoire of $CD4^+$ cells induced by CLIP was less diverse than the repertoire of $CD4^+$ cells in normal mice.

## Results

### Precursor Frequency of H2-$M^-$ $CD4^+$ Cells Reactive to H2-$M^+$ APCs

Although $CD4^+$ cells from H2-$M^-$ mice display strong reactivity to syngeneic H2-$M^+$ APCs in terms of primary MLRs in vitro (Fung-Leung et al., 1996; Martin et al., 1996; Miyazaki et al., 1996), the precise frequency of H2-$M^-$ $CD4^+$ cells that are reactive to H2-$M^+$ APCs has yet to be established. In the H2-$A^b$+Eα peptide transgenic model, Ignatowicz et al. (1996) estimated the precursor frequency of $CD4^+$ cells reactive to syngeneic wild-type APCs to be about 65% based on the frequency of T cell hybridomas generated from these mice that were autoreactive. Extrapolating from these data to H2-$A^b$+CLIP–expressing H2-$M^-$ mice is difficult, however, because the MHC/peptide complexes expressed by the two types of mice differ in terms of structure and expression levels: H2-$A^b$+CLIP complexes are expressed at a level similar to wild-type class II molecules, whereas the H2-$A^b$+Eα complexes in the above transgenic line are expressed at a much lower level (Fung-Leung et al., 1996; Ignatowicz et al., 1996; Martin et al., 1996; Miyazaki et al., 1996).

The precursor frequency of autoreactive $CD4^+$ cells in H2-$M^-$ mice was assessed at the level of naive T cells using two different methods. First, precursor frequency was measured by determining the proportion of $CD4^+$ cells that undergo cell division upon contact with APCs in vitro in the presence of colchicine, as described by Wilson et al. (1968). Since the antimitotic drug colchicine allows entry into cell cycle and DNA synthesis but prevents cell division, the precursor frequency for alloantigens can be estimated by counting numbers of DNA-synthesizing T cells after culture with allogeneic versus syngeneic APCs. Past studies with this technique used [$^3$H]thymidine ([$^3$H]TdR) incorporation followed by autoradiography to detect cells in S phase (Wilson et al., 1968). We modified this method by substituting bromodeoxyuridine (BrdU) for [$^3$H]TdR and using fluorescence-activated cell sorter (FACS) analysis to detect S-phase ($BrdU^+$) cells.

In Figure 1, H2-$M^-$ ($H2^b$) $CD4^+$ cells were cultured with H2-$M^+$ B6 ($H2^b$) versus H2-$M^-$ irradiated splenic APCs in the presence of colchicine and BrdU for 3 days and double-stained for CD4 versus BrdU. As expected, in the presence of H2-$M^-$ APCs, very few H2-$M^-$ $CD4^+$ cells became activated and incorporated BrdU (1.5%; Figure 1, left column, gated on $CD4^+$ cells). In contrast, culture with H2-$M^+$ B6 APCs induced about 40% of the H2-$M^-$ $CD4^+$ cells to undergo DNA synthesis; a slightly lower proportion of H2-$M^-$ $CD4^+$ cells (about 30%) incorporated BrdU in the presence of allogeneic bm12 (H2-$A^{bm12}$) APCs. In control cultures, about 10% of normal B6 $CD4^+$ cells incorporated BrdU against bm12 APCs, relative to less than 1% labeling with syngeneic B6 APCs. In addition to analyzing $CD4^+$ cells for BrdU uptake, the cells were stained for CD69 expression, a marker that is up-regulated upon T cell activation (Yokoyama et al., 1988). As shown in Figure 1 (right column), CD69 up-regulation correlated closely with BrdU incorporation. Similar findings applied to CD25 (interleukin-2 receptor α) expression (data not shown).

Since the above methods rely on inducing overt T cell activation and may not be representative of the conditions encountered in vivo, these methods may underestimate the proportion of T cells with physiological reactivity for wild-type APCs. To quantitate precursor frequency more accurately, H2-$M^-$ $CD4^+$ cells were subjected to blood-to-lymph recirculation through allogeneic versus syngeneic hosts (Atkins and Ford, 1975; Ford et al., 1975; Sprent et al., 1976). This method relies on the fact that contact with specific antigen in vivo causes recirculating T cells to become transiently trapped in regions of antigen concentration and thus to disappear from thoracic duct lymph (Sprent et al., 1971; Rowley et al., 1972). The antigen-reactive T cells are trapped for 1–2 days in the spleen and lymph nodes of the host, while nonreactive cells recirculate freely. Thus, by calculating the fraction of injected cells that fail to recirculate, the frequency of antigen-reactive cells can be measured. With this method, the precursor frequency of rat T cells for MHC-different APCs was calculated to be about 12% (Ford et al., 1975). Precursor frequencies obtained by this method are usually higher than results obtained from other approaches and include cells that do not enter cell cycle. This may reflect that antigen-specific trapping in vivo is highly sensitive and affects T cells with low affinity for antigen (Atkins and Ford, 1975; Gao et al., 1989).

To measure the precursor frequency of H2-$M^-$ $CD4^+$ cells reactive to H2-$M^+$ APCs, a mixture of $40 \times 10^6$ H2-$M^-$ ($H2^b$, Thy-$1.2^+$, Ly $5.2^+$) and $20 \times 10^6$ B6.PL ($H2^b$,

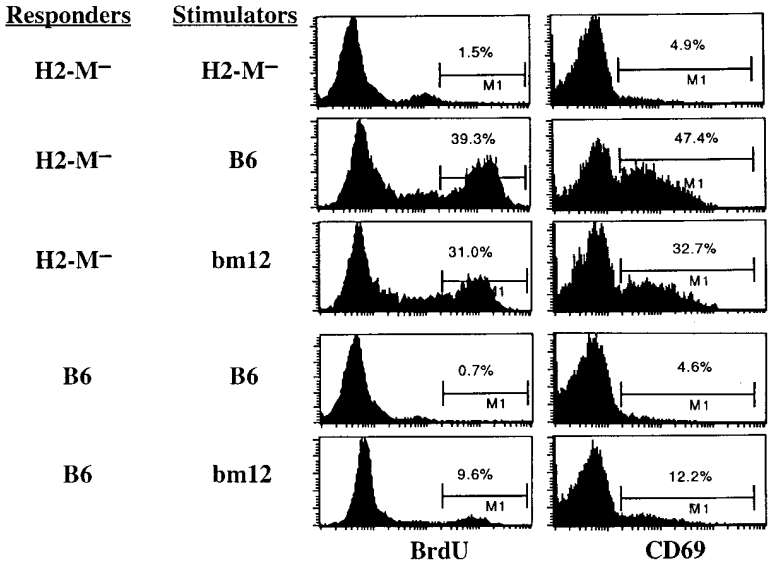


Figure 1. Precursor Frequencies of H2-M$^-$ (H2-A$^b$) CD4$^+$ Cells Reactive to B6 and bm12 APCs In Vitro

LN cells ($2 \times 10^6$) from an H2-M$^-$ mouse and a control B6 were cultured with the indicated T-depleted irradiated spleen cells ($5 \times 10^6$) in 24-well plates for 3 days in the presence of 1 μM colchicine and 5 μg/ml BrdU. Cells were harvested and double stained for CD4 versus BrdU or CD4 versus CD69 and analyzed by FACS. Histograms are shown on gated CD4$^+$ cells. Background staining with an irrelevent antibody was less than 1%. Two other experiments gave similar results.

Thy-1.1$^+$, Ly 5.2$^+$) lymph node (LN) cells was injected into lightly irradiated (600 rad) B6.Ly 5.1 (H2$^b$, Thy-1.2$^+$, Ly 5.1$^+$) mice. One day later, blood from the injected host was collected and peripheral blood lymphocytes (PBLs) were triple-stained for CD4, Thy-1.2, and Ly 5.2. Analysis of PBLs is virtually the same as analyzing cells in thoracic duct lymph since thoracic duct lymph drains directly into the venous system. Gating on the donor (Ly 5.2$^+$) T cells in host PBLs showed that the ratio of H2-M$^-$ (Thy-1.2$^+$) versus B6.PL (Thy-1.2$^-$, Thy-1.1$^+$) cells decreased by an average of 76% (± 7%) (mean of three experiments) relative to the cells prior to injection; a representative experiment is shown in Figure 2A. This finding suggests that a high proportion (76%) of the injected H2-M$^-$ CD4$^+$ cells were selectively removed from the circulation through recognition of host H2-M$^+$ APCs. Consistent with this idea, analyzing the cells harvested from the host spleen revealed a reciprocal 48% increase in the ratio of H2-M$^-$ versus B6.PL CD4$^+$ cells, indicating that the cells were trapped largely in the spleen (Figure 2A). As controls, injection of a mixture of allogeneic bm12 plus B6.PL LN cells into irradiated B6.Ly 5.2 mice resulted in trapping of about 9% of bm12 CD4$^+$ cells (Figure 2B), whereas injection of a mixture of B6 and B6.PL LN cells into irradiated B6.Ly 5.2 mice resulted in no trapping of B6 CD4$^+$ cells (Figure 2C).

### The Repertoire of H2-M$^-$ CD4$^+$ Cells after Depletion of Autoreactive Cells

It is difficult to study the repertoire of H2-M$^-$ CD4$^+$ cells for foreign antigens in H2-M$^-$ mice per se because the APCs in these mice fail to present whole soluble antigens or peptides efficiently (Martin et al., 1996; Miyazaki et al., 1996). Likewise, injecting these mice with normal B6 APCs prior to antigen injection is impractical because these APCs are strongly immunogenic for mature H2-M$^-$ CD4$^+$ cells. Hence, to study the repertoire of H2-M$^-$ CD4$^+$ cells for "nonself" antigens, it is essential first to deplete the T cells of reactivity to B6 APCs. Two approaches were used to remove these cells: by generating H2-M$^+$→H2-M$^-$ bone marrow (BM) chimeras and by depleting B6-reactive T cells by blood-to-lymph recirculation.

### Depletion of Autoreactive Cells in Bone Marrow Chimeras

Since mature H2-M$^-$ CD4$^+$ cells are strongly reactive to normal B6 APCs, exposing immature H2-M$^-$ CD4$^+$ cells to B6 APCs during selection in the thymus would be expected to delete CD4$^+$ cells of reactivity to normal B6 APCs, thus leading to selective survival of CD4$^+$ cells with potential reactivity to other antigens. Thus, H2-M$^+$→H2-M$^-$ chimeras were generated by reconstituting groups of heavily irradiated (1000 cGy) H2-M$^-$ (H2$^b$, Thy-1.2$^+$) hosts with H2-M$^+$ B6.PL (H2$^b$, Thy-1.1$^+$) BM cells ($4 \times 10^6$ T-depleted cells/mouse). In these chimeras, the immature thymocytes derived from normal B6 stem cells will undergo positive selection only on CLIP. However, colonization of the host thymus with donor-derived normal B6 APCs will then induce negative selection of T cells reactive to syngeneic H2-M$^+$ APCs, and the T cells released from the thymus of the chimeras should be depleted of B6 reactivity.

A representative experiment on the phenotype of thymus and LN cells recovered from B6.PL BM→H2-M$^-$ chimeras with the control H2-M$^-$ BM→H2-M$^-$ and B6.PL BM→H2-M$^+$ (H2-M$^{+/-}$) chimeras is shown in Figure 3; data on total recoveries of thymocyte subsets are summarized in Table 1. Relative to the control B6.PL BM→H2-M$^+$ chimeras, it can be seen that total numbers of CD4$^+$8$^-$ thymocytes were reduced by 55% in H2-M$^-$ BM→H2-M$^-$ chimeras and by 91% in B6.PL BM→H2-M$^-$ chimeras (Table 1). Numbers of CD4$^+$8$^-$ thymocytes were thus about 5-fold lower in B6.PL BM→H2-M$^-$ chimeras (negative selection to H2-A$^b$ APCs) than in H2-M$^-$ BM→H2-M$^-$ chimeras (no negative selection to H2-A$^b$ APCs).

In the case of CD4$^-$CD8$^+$ thymocytes, total numbers of these cells in B6.PL BM→H2-M$^-$ and B6.PL BM→H2-M$^+$ chimeras were similar (Table 1). Unexpectedly, however, numbers of CD4$^-$CD8$^+$ thymocytes were considerably increased in H2-M$^-$ BM→H2-M$^-$ chimeras. The

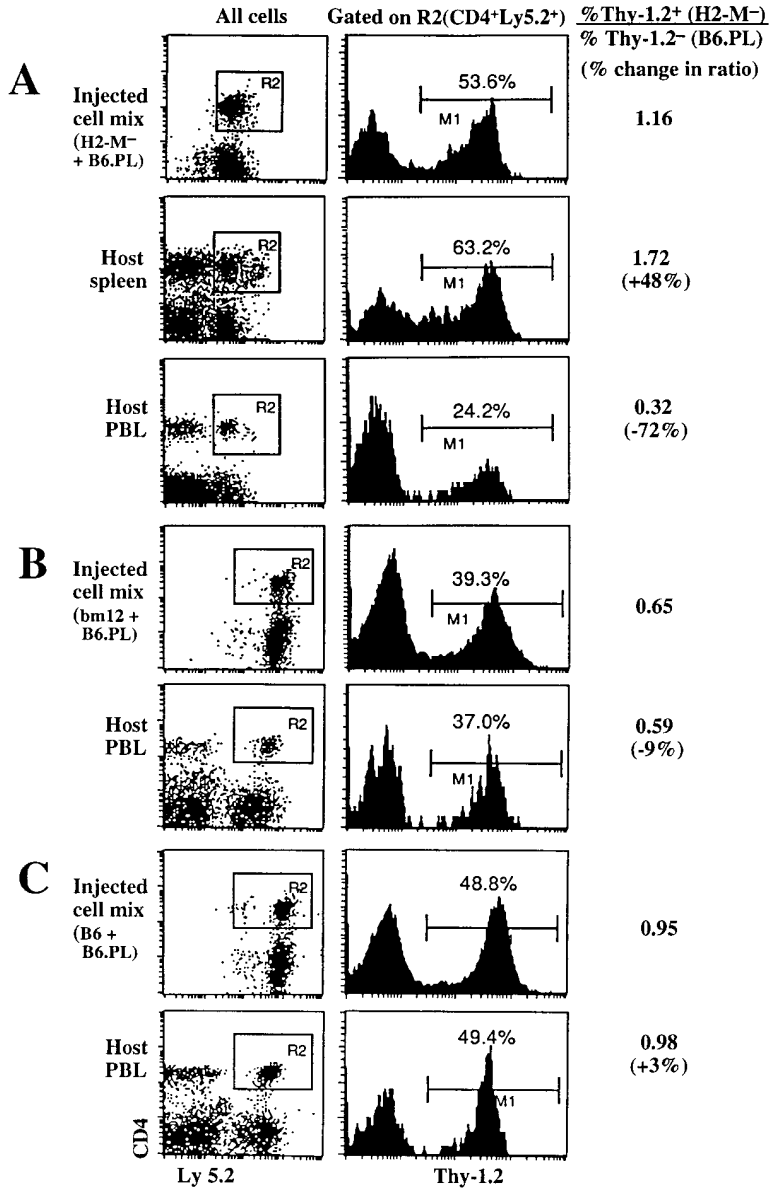


Figure 2. Measurement of Precursor Frequencies of Host-Reactive CD4+ T Cells by Blood-to-Lymph Recirculation

(A) To measure the precursor frequency of H2-M− CD4+ cells autoreactive to syngeneic normal B6 APCs, a mixture of H2-M− CD4+ (Thy-1.2+, Ly 5.2+) and B6.PL (Thy-1.1+, Ly 5.2+) LN cells was injected into irradiated (600 cGy) B6.Ly 5.1 (Thy-1.2+, Ly 5.2−) mice. Lymphocytes from the host spleen and blood were collected 24 hr later and triple stained for CD4, Ly5.2 and Thy-1.2; the ratios of H2-M− CD4+ versus B6.PL CD4+ cells in these preparations were then compared to the injected cell population. Using these ratios, the percent change in the donor-type H2-M− CD4+ cells in blood (the precursor frequency of reactive cells) was calculated by 1 − (ratio in the host PBL/ratio of injected mixture).

(B and C) As controls, the precursor frequencies of bm12 CD4+ cells reactive to B6 APCs (B) and B6 CD4+ cells reactive to B6 APCs (C) were calculated. Using a similar approach as in (A), a mixture of bm12 (Thy-1.2+, Ly 5.2+) and B6.PL (Thy-1.1+, Ly 5.2+) or B6 (Thy-1.2+, Ly 5.2+) and B6.PL (Thy-1.1+, Ly 5.2+) LN cells was injected into B6.Ly 5.1 (Thy-1.2+, Ly 5.2−) hosts, and the ratio of bm12 versus B6.PL CD4+ cells in the host was analyzed the next day. Two other experiments gave similar findings.

simplest explanation for this finding is that the CD4−CD8+ cells generated in B6.PL BM→H2-M− and B6.PL BM→H2-M+ chimeras underwent significant negative selection to the class II–associated peptides present on normal B6 APCs; such negative selection is absent in H2-M− BM→H2-M− chimeras, thus leading to overproduction of CD8+ cells. This interpretation is in line with the observation that normal CD8+ cells have appreciable reactivity for class II alloantigens (Gao et al., 1989) and class II–associated endogenous superantigens (Webb and Sprent, 1990) when supplemented with exogenous help. In addition, preliminary work has shown that the CD8+ cells generated in H2-M− mice give much stronger responses to class II alloantigens in vitro than CD8+ cells from normal mice (data not shown). This also applies to the CD8+ cells generated in class II− mice (Shimizu and Takeda, 1997).

The important finding in the chimeras is that total numbers of CD4+8− thymocytes were reduced by about 80% in B6.PL BM→H2-M− chimeras relative to H2-M− BM→H2-M− chimeras (Table 1), suggesting that exposure to normal B6 APCs during thymocyte generation eliminated 80% of the cells undergoing positive selection to CLIP. This finding agrees closely with the data in Figure 2, which shows that about 75% of H2-M− CD4+ cells were autoreactive to B6 APCs by the blood-to-lymph recirculation method. Negative selection in the thymus to B6 APCs in B6.PL BM→H2-M− chimeras seemed to occur in the medulla, since histological staining for apoptotic cells using the TUNEL method (Surh and Sprent, 1994) revealed increased numbers of apoptotic cells in the medulla of B6.PL BM→H2-M− chimeras as compared to the control H2-M− BM→H2-M− thymi (data not shown); the frequency of apoptotic cells in the thymic cortex of the two types of chimeras was similar.

For the control H2-M−→H2-M− chimeras, the CD4+8−

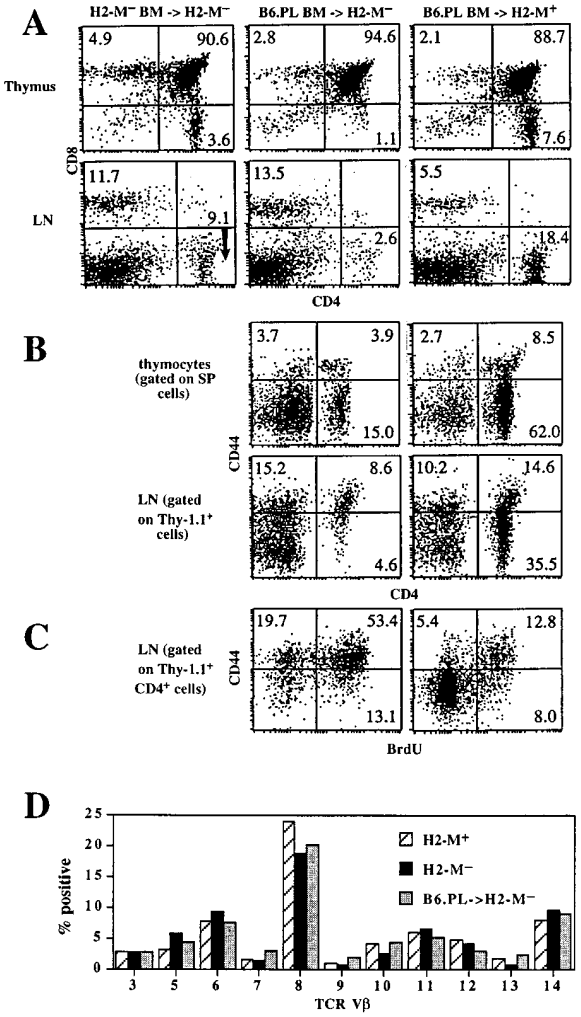


Figure 3. The Phenotype of CD4$^+$ Cells Developing in H2-M$^-$→H2-M$^-$, B6.PL (H2-M$^+$)→H2-M$^-$, and B6.PL→H2-M$^+$ (H2-M$^{+/-}$) BM Chimeras

BM chimeras were generated by reconstituting host mice exposed to a lethal dose of irradiation (1000 cGy) with 4 × 10$^6$ T-depleted BM cells. The chimeras were analyzed at least 4 weeks after BM cell reconstitution.

(A) Thymocytes and LN cells from B6.PL (H-2$^b$, Thy-1.1$^+$) BM→H2-M$^-$ (H-2$^b$, Thy-1.2$^+$) chimeras were stained for CD4, CD8, and Thy-1.2 and analyzed by FACS. All thymocytes (>99%) and 50–70% of LN CD4$^+$ cells from these chimeras were Thy-1.2$^-$ (donor-type); host-type (Thy-1.2$^+$) cells were excluded in the LN analysis in the B6.PL BM→H2-M$^-$ and B6.PL BM→H2-M$^+$ chimeras. Control H2-M$^-$ BM→H2-M$^-$ and B6.PL BM→H2-M$^+$ chimeras were analyzed at the same time. The total thymocyte recoveries from the three types of chimeras were similar (Table 1).

(B) The phenotype of CD4$^+$ cells in B6.PL BM→H2-M$^-$ versus B6.PL BM→H2-M$^+$ chimeras was analyzed by triple staining thymocytes for CD4, CD8, and CD44 and analyzed by FACS on gated SP (CD4$^+$8$^-$ and CD4$^-$8$^+$) cells; LN cells were triple stained for CD4, CD44, and Thy-1.1 and gated for Thy-1.1$^+$ cells. Similar findings were also observed when cells were stained for CD45RB (not shown).

(C) The turnover rate of CD4$^+$ cells in the B6.PL BM→H2-M$^-$ versus B6.PL BM→H2-M$^+$ chimeras was analyzed by giving BrdU in drinking water for 9 days and then staining the LN cells for BrdU, CD4, Thy-1.1, and CD44. The four-color–stained cells were analyzed by FACS and gated on the Thy-1.1$^+$ CD4$^+$ population.

(D) LN cells from H2-M$^+$ (H2-M$^{+/-}$) and H2-M$^-$ mice and B6.PL BM→H2-M$^-$ chimeras were double-stained for CD4 and various TCR Vβ chains. The percentages of CD4$^+$ cells bearing each Vβ chain are shown.

cells arising in the thymus and LN of these chimeras consisted predominantly of naive-phenotype (CD44$^{lo}$CD45RB$^{hi}$) cells, as expected (data not shown). Similar findings applied to B6.PL→H2-M$^+$ chimeras (Figure 3B). Surprisingly, this was not the case for the B6.PL BM→H2-M$^-$ chimeras. Thus, in more than 20 such chimeras examined, the LN CD4$^+$ cells in all of these mice contained a high proportion of cells with an activated CD44$^{hi}$CD45RB$^{lo}$ phenotype (Figure 3B and data not shown). Most of these cells incorporated BrdU over a 9-day labeling period (Figure 3C), indicating that the cells were cycling. However, CD69 and CD25 expression was low (data not shown), implying a chronic form of low-level stimulation. Significantly, expression of an activated phenotype applied neither to the donor-derived CD8$^+$ cells nor to residual host T cells (Figure 3B and data not shown), suggesting that activation of the donor-derived CD4$^+$ cells was not simply a nonspecific response to stress and/or infection. Activation of CD4$^+$ cells in B6.PL BM→H2-M$^-$ chimeras seemed to occur in the peripheral lymphoid tissues because most of the CD4$^+$8$^-$ thymocytes in these mice expressed a naive phenotype (Figure 3B).

To examine the repertoire of CD4$^+$ cells generated in the chimeras, CD4$^+$ cells were typed for TCR Vβ usage. As shown in Figure 3D, the donor BM-derived CD4$^+$ cells from the B6.PL BM→H2-M$^-$ chimeras displayed a Vβ profile similar to that of the CD4$^+$ cells from normal and H2-M$^-$ mice (Figure 3D).

### Responses to Alloantigens

T cell proliferation to allogeneic APCs is considered to reflect a polyclonal response against a spectrum of diverse peptides present on allogeneic MHC molecules (Sherman and Chattopadhyay, 1993). Thus, the capacity of CD4$^+$ cells from B6.PL BM→H2-M$^-$ chimeras to respond to bm12 and other allogeneic APCs in MLRs is an indicator of T cell diversity. The donor-type CD4$^+$ cells from B6.PL BM→H2-M$^-$ chimeras were purified by killing CD8$^+$, B and Thy-1.2$^+$ host T cells (with monoclonal antibodies [MAbs] plus C′) followed by panning on anti-CD4–coated plates. These cells were unresponsive to B6 APCs (as expected) but gave only very low responses to allogeneic bm12, B10.BR, and B10.D2 APCs (not shown). However, these cells also failed to respond to anti-CD3 or concanavalin A (not shown). This finding was not surprising considering that the majority of the chimeric CD4$^+$ cells had an activated phenotype; these cells are known to respond very poorly to stimulation through the TCR, at least in vitro (Byrne et al., 1988). To overcome this problem, resting-phenotype CD45RB$^{hi}$, Thy-1.1$^+$ CD4$^+$ cells from B6.PL BM→H2-M$^-$ chimeras were purified by FACS sorting. Significantly, these cells did proliferate to allogeneic bm12 APCs (but not to B6) in MLRs at levels nearly comparable to CD45RB$^{hi}$CD4$^+$ cells sorted from control B6.PL BM→H2-M$^+$ chimeras (Figure 4); in contrast to CD45RB$^{hi}$ cells,

Table 1. Production of Thymocyte Subsets in Irradiation BM Chimeras

| Cellularity of Thymus | Chimeras Examined (% Recovery) | | |
|---|---|---|---|
| | B6.PL BM→H2-M$^+$ | H2-M$^-$ BM→H2-M$^-$ | B6.PL BM→H2-M$^-$ |
| Total cells ($\times 10^{-6}$) | 155 | 154 | 115 |
| CD4$^+$CD8$^-$ (%) | 8.2 | 3.7 | 1.0 |
| CD4$^-$CD8$^+$ (%) | 2.2 | 5.0 | 3.5 |
| CD4$^+$CD8$^+$ (%) | 87.8 | 90.2 | 93.1 |
| CD4$^+$CD8$^-$ count ($\times 10^{-6}$) | 12.7 (100%) | 5.7 (45%) | 1.2 (9%) |
| CD4$^-$CD8$^+$ count ($\times 10^{-6}$) | 3.4 (100%) | 7.7 (226%) | 4.0 (118%) |

Chimeras were established by intravenously injecting 4 $\times$ 10$^6$ T cell–depleted BM cells into 6- to 8-week-old host mice exposed to a 1000 cGy dose of irradiation. Data shown are average percentages of positive cells as determined by FACS analysis on three to four mice per group, which were analyzed individually 4–6 weeks after BM reconstitution. The percent change in SP cell counts as compared to the counts obtained from B6.PL BM→H2-M$^+$ (H2-M$^{+/-}$) chimeras are noted in parentheses.

no responses were observed with CD45RB$^{lo}$ cells from B6.PL BM→H2-M$^-$ chimeras (Figure 4).

To confirm that the alloresponse observed with naive-phenotype chimeric CD4$^+$ T cells is due to a polyclonal T cell response, TCR V$\beta$ expression on the responding CD4$^+$ cells was measured. This was analyzed by preparing CD4$^+$ cells from B6.PL BM→H2-M$^-$ versus H2-M$^+$ chimeras, enriching these cells for resting-phenotype cells by depleting CD44$^{hi}$ cells and then culturing the T cells with T-depleted bm12 APCs in the presence of colchicine, as described above. After 2 days, TCR usage on the bm12-reactive T cells was analyzed by triple staining cells for CD69, CD4, and TCR V$\beta$ and V$\alpha$ expression. As shown in Figure 4B, gating on activated (CD69$^{hi}$) cells revealed that the bm12-reactive CD4$^+$ cells from the B6.PL BM→H2-M$^-$ chimeras expressed multiple TCR $\beta$ and $\alpha$ (V$\alpha$2) chains in frequencies similar to CD4$^+$ cells from control B6.PL BM→H2-M$^+$ chimeras. These data suggest that CD4$^+$ cells in B6.PL BM→H2-M$^-$ chimeras do have a polyclonal repertoire similar to that of normal B6 CD4$^+$ cells, at least in terms of response to alloantigens.

### Depletion of B6-Reactive H2-M$^-$ CD4$^+$ Cells by Blood-to-Lymph Recirculation

One problem with the above data on sorted CD45RB$^{hi}$CD4$^+$ cells is that these cells constitute only a minor fraction of total CD4$^+$ cells and thus may not be representative of all donor-derived CD4$^+$ cells in the B6.PL BM→H2-M$^-$ chimeras. To circumvent this problem, B6-depleted H2-M$^-$ CD4$^+$ cells were obtained through use of the blood-to-lymph recirculation method as described above; these cells would be expected to consist almost entirely of naive-phenotype cells.

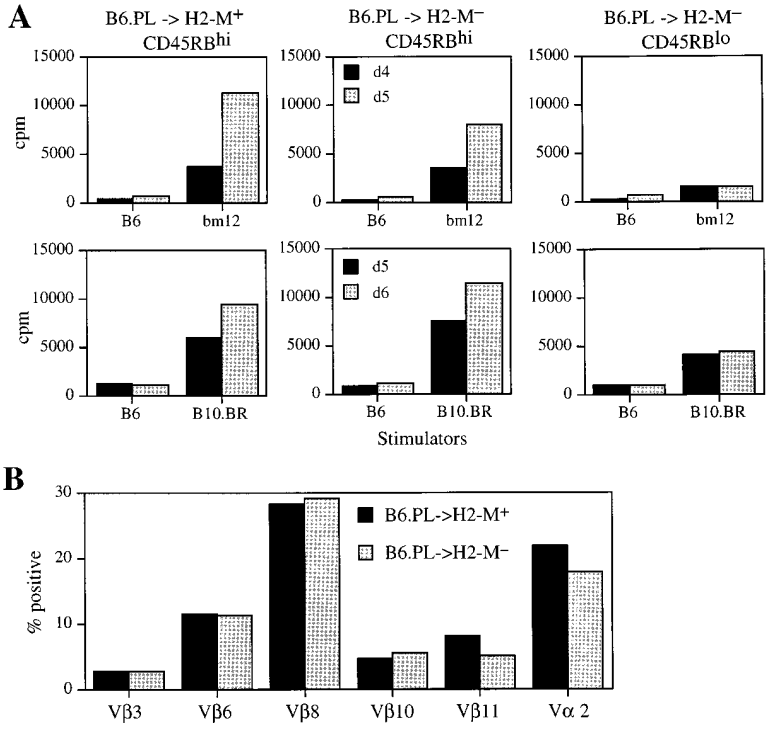


Figure 4. Alloreactive Responses by CD4$^+$ Cells from B6.PL→H2-M$^-$ Chimeras

(A) Proliferative responses of FACS-sorted CD45RB$^{hi}$ versus CD45RB$^{lo}$ CD4$^+$ LN cells from B6.PL→H2-M$^-$ versus B6.PL→H2-M$^+$ (H2-M$^{+/-}$) chimeras to bm12 and B10.BR allogeneic spleen cells were measured in MLR cultures. Cells were sorted by staining LN cells with CD45RB and CD4 after depletion of CD8$^+$, B, and host Thy-1.2$^+$ cells with a cocktail of MAbs plus C′. Sorted cells (1 $\times$ 10$^5$/well) were incubated with T-depleted stimulator spleen cells for time shown, and the cultures were pulsed with [$^3$H]TdR (1 $\mu$Ci/well) for 18 hr prior to harvest.

(B) To measure TCR V chain usage on the CD4$^+$ cells responding to allogenic bm12 stimulator cells, purified CD4$^+$ cells from B6.PL→H2-M$^-$ versus B6.PL→H2-M$^+$ (H2-M$^{+/-}$) chimeras were incubated in MLRs with T-depleted bm12 spleen cells in the presence of 1 $\mu$M colchicine for 2 days and triple stained for CD4, CD69, and indicated TCR V$\alpha$ and V$\beta$ chains. The TCR V expression data shown are on gated CD4$^+$CD69$^{hi}$ cells; CD69$^{hi}$ cells were found to be bm12-responding T cells, since their expression level of CD69 was considerably higher than the preexisting CD69$^+$ cells. Before culture, the purified CD4$^+$ cells from B6.PL→H2-M$^-$ chimeras were depleted of CD44$^{hi}$ cells using magnetic columns as described in Experimental Procedures.

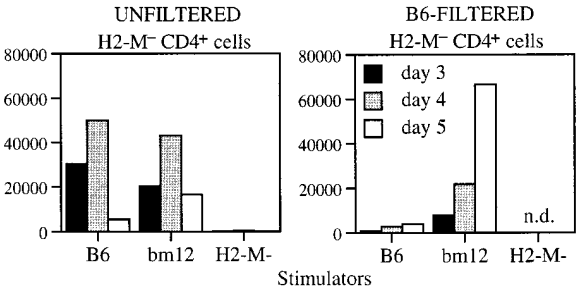


Figure 5. Alloreactivity of H2-M$^-$ CD4$^+$ Cells after Depletion of Autoreactivity to B6 APC

MLR responses of unmanipulated H2-M$^-$ CD4$^+$ cells were compared with H2-M$^-$ CD4$^+$ cells depleted of autoreactivity by blood-to-lymph recirculation. B6-"filtered" H2-M$^-$ CD4$^+$ cells were obtained by injecting large numbers of H2-M$^-$ LN cells into irradiated B6.PL hosts and collecting thoracic duct lymph (TDL) during the next 20–40 hr period. Cells from TDL were depleted of CD8$^+$, B, and host Thy-1.1$^+$ T cells with a cocktail of MAbs plus C′ prior to culture with the indicated stimulators. MLR responses were measured by culturing $1 \times 10^5$ purified CD4$^+$ cells with T-depleted irradiated stimulator spleen cells for 3, 4, or 5 days; cultures were pulsed with [$^3$H]TdR (1 μCi/well) for 18 hr prior to harvest. n.d., not done.

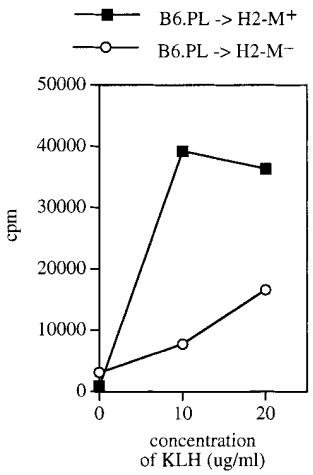


Figure 6. Response of B6.PL → H2-M$^-$ Chimeras to KLH

Chimeras were injected in the foot pad and at the base of the tail with 50 μg of KLH emulsified in CFA. Seven days later, the draining lymph node cells were cultured with KLH. Cultures were incubated for 5 days; [$^3$H]TdR was added for the last 18 hr. To partially compensate for differential LN CD4$^+$ counts in the two types of chimeras, LN cells from B6.PL → H2-M$^-$ chimeras were used at a 2-fold higher dose ($8 \times 10^5$/well) than LN cells from B6.PL → H2-M$^+$ (H2-M$^{+/-}$) chimeras.

Large numbers of H2-M$^-$ LN cells were injected intravenously into irradiated B6.PL hosts, and thoracic duct lymphocytes were harvested during the next 24–48 hr period; as shown earlier (Figure 2), in this system the host B6-reactive CD4$^+$ cells become trapped in areas enriched for host APCs, such as the spleen, while nonreactive cells circulate freely. The B6-filtered H2-M$^-$ CD4$^+$ cells, which were nearly all naive-phenotype (data not shown), were then depleted of CD8$^+$, B, and host Thy-1.1$^+$ cells (with MAbs plus C′) and used in MLRs against B6 versus bm12 APCs. As shown in Figure 5, the filtered CD4$^+$ cells proliferated strongly to bm12 APCs but not to B6 APCs, whereas undepleted H2-M$^-$ CD4$^+$ cells proliferated strongly to both B6 and bm12 APCs. This finding corroborates the above data obtained from sorted CD45RB$^{hi}$ chimera CD4$^+$ cells, and, more important, indicates that the naive-phenotype CD4$^+$ cells in B6.PL BM→H2-M$^-$ chimeras closely resemble the subset of B6-unresponsive CD4$^+$ cells found in normal H2-M$^-$ mice.

### Reactivity against Soluble Foreign Antigens

To obtain further evidence that the repertoire of B6-tolerant H2-M$^-$ CD4$^+$ cells for foreign antigens is polyclonal, we analyzed the responses of B6.PL BM→H2-M$^-$ chimeras upon immunization with defined soluble antigens. The chimeras were injected separately with two commonly used protein antigens, keyhole limpet hemocyanin (KLH) and fowl γ-globulin (FGG) in complete Freund's adjuvant (CFA) in the foot pad and the base of the tail; 7–10 days later the draining LN cells were tested for proliferative responses to the immunizing antigen in vitro. Figure 6 shows that, relative to control B6.PL→H2-M$^+$ chimeras, immunization of B6.PL→H2-M$^-$ chimeras with KLH resulted in low but significant proliferative responses to KLH upon secondary challenge in vitro; similar results were observed for FGG (data not shown). This finding is significant because the proportions of CD4$^+$ cells in the LN from the B6.PL→H2-M$^-$ chimeras were 4-fold lower than in the control B6.PL BM→H2-M$^+$ chimeras.

### Capacity of H2-M$^-$ Mice to Positively Select TCR Transgenic T Cells

Production of large numbers of CD4$^+$ cells in H2-M$^-$ mice implies that a significant proportion of DP thymocytes have sufficient reactivity for H2-A$^b$+CLIP to become positively selected. This conclusion raises the question as to whether DP thymocytes from TCR transgenic mice would be able to undergo positive selection to H2-A$^b$+CLIP. To examine this question, groups of H2-M$^-$ (H2-A$^b$) mice were lethally irradiated (1000 cGy) and reconstituted with BM cells taken from two TCR transgenic mice: AND (anti-pigeon cytochrome C) and DO11 (anti-ovalbumin) (Kaye et al., 1989; Murphy et al., 1990). When the chimeras were examined 5–7 weeks after reconstitution, large numbers of CD4$^+$8$^-$ thymocytes were generated when the transgenic BM cells were used to reconstitute H2-M$^+$ (H2-A$^b$) littermates. Gating on single-positive (SP) thymocytes indicated that the majority of the CD4$^+$8$^-$ thymocytes in these control chimeras were clonotype positive (Vα11$^+$ for AND, and KJ1–26$^+$ for DO11) (Figure 7). In marked contrast, clonotype-positive CD4$^+$8$^-$ thymocytes were almost undetectable in H2-M$^-$ (H2-A$^b$) hosts (Figure 7). Similar findings applied at the level of CD4$^+$ LN cells (data not shown). Exposure to H2-A$^b$+CLIP in the thymus was thus unable to cause positive selection of TCR transgenic CD4$^+$ cells.

### Discussion

The observation that H2-M$^-$ mice appear to express only a single MHC class II–bound peptide, CLIP, makes

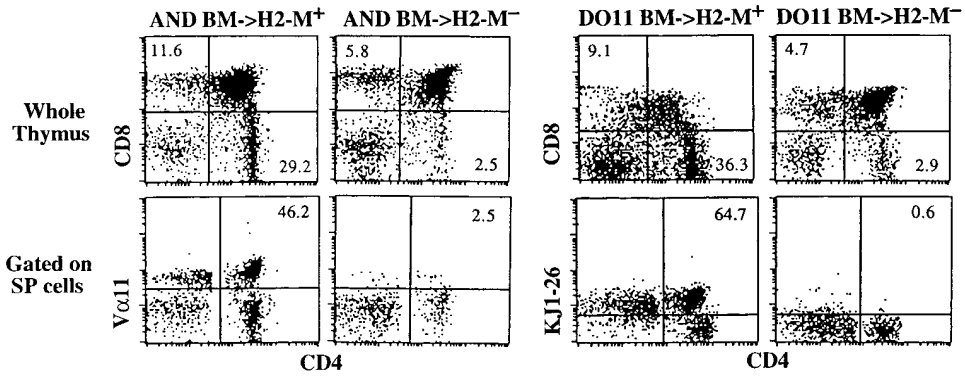


Figure 7. Failure of H2-M$^-$ Mice to Positively Select TCR Transgenic T Cells

Groups of H2-M$^+$ (H2-M$^{+/-}$) and H2-M$^-$ (H2-A$^b$) mice were lethally irradiated (1000 cGy) and reconstituted with BM cells from two TCR transgenic lines that are known to be positively selected in B6 (H2-M$^+$, H2-A$^b$) mice: AND (anti-pigeon cytochrome C) and DO11 (anti-ovalbumin). Thymocytes from the chimeras were triple stained at 5 weeks post-reconstitution for CD4, CD8, and the clonotypic TCR (V$\alpha$11$^+$ for AND, and KJ1–26$^+$ for DO11) and analyzed by FACS.

these mice a useful tool for examining the influence of individual peptides in positive selection of CD4$^+$ T cells. Studying the repertoire diversity of H2-M$^-$ CD4$^+$ cells is complicated by the fact that CLIP expression on the APCs of these mice prevents binding of either self-peptides or foreign peptides. For this reason, the response of CD4$^+$ cells to foreign antigens cannot be measured in unmanipulated H2-M$^-$ mice. In addition, the failure of H2-M$^-$ APCs to present self-peptides precludes self-tolerance induction, with the result that the repertoire of CD4$^+$ cells generated in H2-M$^-$ mice is strongly skewed to self-components. In this report, we show that the proportion of H2-M$^-$ CD4$^+$ cells with reactivity for the self-components on normal wild-type APCs is extremely high, at 70–80%. More important, depleting H2-M$^-$ CD4$^+$ cells of reactivity to self-components leaves a minor subset of CD4$^+$ cells that is polyclonal in terms of V$\beta$ diversity and responds well to foreign antigens.

The values obtained for the precursor frequency of H2-M$^-$ CD4$^+$ cells for wild-type APCs varied according to the method used. When H2-M$^-$ CD4$^+$ cells were cultured with wild-type APCs in vitro, about 40% of the CD4$^+$ cells were responsive in terms of BrdU incorporation and up-regulation of CD69 and CD25. However, a substantially higher proportion, 70–80%, of the cells were reactive as measured by recirculation through wild-type mice and formation of T cells in H2-M$^+$→H2-M$^-$ BM chimeras. These two methods rely on selective T cell trapping in the lymphoid tissues and intrathymic tolerance induction, respectively, and are thus much more sensitive for detecting physiological reactivity to antigen than the in vitro techniques.

The finding that about 75% of H2-M$^-$ CD4$^+$ cells had detectable reactivity for wild-type APCs is in close accord with the data of Ignatowicz et al. (1996) on transgenic mice expressing a single species of H2-A$^b$ + E$\alpha$. As mentioned earlier, these workers found that 65% of T hybridomas prepared from CD4$^+$ spleen cells of these mice had reactivity to wild-type APCs in terms of interleukin-2 production. The close correlation in the proportion of autoreactive cells in H2-M$^-$ and H2-A$^b$ + E$\alpha$ mice is noteworthy because the peptides controlling positive selection in these two lines are different; moreover, the density of MHC/peptide complexes is much lower in the H2-A$^b$ + E$\alpha$ line than in H2-M$^-$ mice. However, each transgenic line selects a substantial repertoire of CD4$^+$ cells with strong autoreactivity. The implication therefore is that in the normal thymus, a high proportion (70–80%) of immature thymocytes with the potential to undergo positive selection to individual peptides is destroyed by negative selection to other peptides prior to export from the thymus (Ignatowicz et al., 1996).

The site of negative selection requires comment. In H2-M$^+$→H2-M$^-$ BM chimeras, negative selection is directed to the various self-peptides expressed on the class II$^+$ donor-derived cells entering the host thymus. Since these cells are prominent only in the medulla and not the cortex, negative selection presumably occurs exclusively in the medulla and the corticomedullary junction. Thymic selection in the chimeras is thus strictly compartmentalized with positive selection to a single peptide, CLIP, occurring in the cortex and negative selection to the normal array of peptides expressed on BM-derived APCs taking place in the medulla. In the normal thymus, by contrast, the majority of the self-peptides expressed on APCs are coexpressed on cortical epithelial cells (Marrack et al., 1993). Hence, negative selection in the normal thymus could occur largely in the cortex concomitant with positive selection. Although negative selection can occur in the cortex in certain transgenic models (Kisielow et al., 1988; Sha et al., 1988; Pircher et al., 1989), whether this also applies to the normal thymus is still questionable. By contrast, the medulla is a well-established site for negative selection (Surh and Sprent, 1994).

It is noteworthy that the few CD4$^+$ cells generated in H2-M$^+$ > H2-M$^-$ BM chimeras consisted predominantly of memory-phenotype (CD44$^{hi}$) cells. This applied to spleen and LN CD4$^+$ cells but not to CD4$^+$8$^-$ thymocytes, implying that the cells switched to a memory phenotype only after leaving the thymus. Why the cells developed this phenotype is still unclear. A nonspecific response to stress or infection did not seem to be involved, because expression of a memory phenotype

applied only to $CD4^+$ cells and not to $CD8^+$ cells. Since most of the memory-phenotype $CD4^+$ cells incorporated BrdU in vivo, division of these cells may reflect a homeostatic mechanism (Rocha et al., 1989) or possibly incomplete tolerance to donor APCs. Although lack of self-tolerance deserves consideration, it is important that both the $CD45RB^-$ and the $CD45RB^+$ subsets of $CD4^+$ cells from the chimeras were unresponsive to normal B6 APCs in vitro.

Since the memory-phenotype cells responded poorly in vitro, examining the responsiveness of $CD4^+$ cells from the chimeras necessitated cell sorting to select for the minor subset of naive-phenotype cells. The significant finding was that this subset of $CD4^+$ cells gave near-normal proliferative responses to alloantigens (bm12 and B10.BR spleen cells) but remained unresponsive to "self" B6 APCs. Similar findings applied when $CD4^+$ cells from nonchimeric H2-$M^-$ mice were depleted of B6 reactivity by blood-to-lymph recirculation through wild-type B6 mice. In each situation the $CD4^+$ cells were polyclonal in terms of Vβ usage.

In addition to responding to alloantigens, the $CD4^+$ cells in H2-$M^+$→H2-$M^-$ BM chimeras gave low but significant proliferative responses to two protein antigens, KLH and FGG, after in vivo priming. Combined with the data on MLR and Vβ usage, these findings suggest that the few $CD4^+$ cells generated in the chimeras had an extensive repertoire for foreign antigens. Despite these findings, it is striking that crossing two H2-$A^b$–restricted TCR transgenic lines to the H2-$M^-$ background failed to cause positive selection. This observation suggests that the repertoire of $CD4^+$ cells in H2-$M^-$ mice contains significant "holes" and is therefore less complete than the repertoire generated in the normal thymus. Hence, it is likely that examining the fine specificity of $CD4^+$ cells responding to individual protein antigens would reveal that the repertoire of H2-$M^-$ $CD4^+$ cells is appreciably less diverse than the repertoire of normal $CD4^+$ cells. We are currently investigating this question.

Collectively, the data in this report support the view that generation of an extensive repertoire of mature $CD4^+$ cells through positive selection requires contact with multiple peptides. Through low-avidity TCR interaction, each individual peptide selects a substantial cohort of $CD4^+$ cells with reactivity for a broad range of antigens. However, the repertoires selected by individual peptides contain significant holes and are therefore not fully diverse. In addition, as a result of negative selection the total number of $CD4^+$ cells undergoing positive selection to individual peptides is limited (10-fold below normal for CLIP in H2-$M^+$→H2-$M^-$ chimeras). Formation of a large pool of $CD4^+$ cells with a fully diverse repertoire thus requires positive selection to multiple peptides.

## Experimental Procedures

### Mice and Chimeras

H2-M-deficient ($H2^b$) and TCR transgenic mice were bred and maintained at the Scripps Research Institute. AND is a B6 transgenic strain expressing TCR specific for a PCC peptide/$E^k$ (Kaye et al., 1989), and DO11.10 is a BALB/c transgenic strain expressing TCR specific for an OVA peptide/$A^d$ (Murphy et al., 1990); this line also undergoes positive selection in H2-$A^b$ mice (Laufer et al., 1996). B6, B6.PL, B6.Ly 5.1, bm12, and B10.BR mice were either obtained from the Scripps Research Institute breeding colony or purchased from the Jackson Laboratory (Bar Harbor, ME).

### Antibodies and Staining Reagents

The following MAbs have been described previously (Kosaka et al., 1992; Sprent et al., 1994; Kishimoto et al., 1995): GK1.5 (anti-CD4), 3.168 (anti-CD8), 19E12 (anti-Thy-1.1), J1j (anti-Thy-1.2), T-24 (anti-Thy-1), J11d (anti-HSA), and 104–2.1 (anti-Ly 5.2). Cy5-conjugated anti-CD4 was produced by labeling protein A–purified GK1.5 with Cy5 using the CyDye FluoroLink-Ab labeling kit (Amersham Life Science, Arlington Heights, IL). The following MAbs and reagents were purchased: fluorescein isothiocyanate (FITC)–conjugated anti-BrdU, phycoerythrin (PE)–conjugated anti-CD4 (Becton Dickinson, San Jose, CA); biotinylated anti-CD8, anti-CD25, anti-CD44, anti-CD45RB, anti-CD69, anti-TCR chains (Vβ 3, 5, 6, 7, 8.1+8.2+8.3, 9, 10, 11, 12, 13, 14, and Vα 2, 11), PE-conjugated anti-CD44, and anti-CD45RB (Pharmingen, San Diego, CA); FITC-labeled anti-CD8, R613-conjugated anti-Thy-1.2, PE- and R670-labeled streptavidin (GIBCO–BRL, Gaithersburg, MD); FITC-labeled anti-mouse immunoglobulin G (γ-specific) antibody and streptavidin (Jackson ImmunoResearch, West Grove, PA); and propidium iodide (PI) and BrdU (Sigma, St. Louis, MO). MAb KJ1–26 (anti-DO11 TCR) was generously provided by P. Marrack.

### Generation of Chimeras

Chimeras were generated by injecting 2–4 × $10^6$ T-depleted BM cells into lethally irradiated (1000 cGy) hosts as previously described (Gao et al., 1990). T cells in the BM were depleted by killing with anti-Thy-1 MAbs (J1j or T-24) plus C′.

### FACS Analysis and Sorting

Cells were stained for two-, three-, or four-color FACS analysis as previously described (Tough and Sprent, 1994; Ernst et al., 1995). For example, to analyze B6.PL→H2-$M^-$ chimeras, cells were triple stained by incubation with a cocktail of FITC-labeled anti-CD8, PE-labeled anti-CD4, R613-conjugated anti-Thy-1.2 MAbs, and PI. To examine memory markers, cells were triple stained by incubation with a cocktail of biotinylated anti-Thy-1.1, PE-conjugated anti-CD44, and Cy5-conjugated anti-CD4 MAbs; washed; and then incubated with FITC-labeled steptavidin plus PI and analyzed on FACSort. BrdU-labeled cells were stained as previously decribed (Tough and Sprent, 1994; Ernst et al., 1995). For example, four-color staining on BrdU-labeled cells was performed by first incubating cells with a mixture of biotinylated anti-Thy-1.1, Cy5-labeled anti-CD4, and PE-conjugated anti-CD44 MAbs; washed and incubated with Red 670-conjugated steptavidin; washed; fixed; treated with DNase; and then incubated with FITC-labeled anti-BrdU MAb as described (Tough and Sprent, 1994). Mice were labeled with BrdU for 9 days by giving BrdU (0.8 mg/ml) in the drinking water as described (Tough and Sprent, 1994). To measure BrdU-labeled cells in MLR cultures, cells were stained with PE-conjugated anti-CD4 MAb, fixed, and then stained with FITC-conjugated anti-BrdU MAb as described (Tough and Sprent, 1994). All stained cells were analyzed by a FACScan or FACSort flow cytometer as described; dead cells were excluded using PI in unfixed cells and by forward/side scatter gating in fixed cells (Tough and Sprent, 1994; Ernst et al., 1995). For sorting naive, donor-phenotype $CD4^+$ cells in the BM chimeras, pooled LN, and spleen cells were first treated with a cocktail of anti-HSA, anti-Thy-1.2, and anti-CD8 MAbs plus C′; washed; and then stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD45RB MAbs with PI, and viable $CD4^+CD45RB^{hi}$ cells were sorted on a FACSVantage.

### In Vitro T Cell Responses

MLRs with sorted or in vivo–filtered cells were performed as described (Sprent and Schaefer, 1985). In brief, 0.5–1 × $10^5$ LN cells were cultured with 7 × $10^5$ irradiated stimulator T-depleted spleen cells in 96-well plates for 3–6 days; cells were pulsed with [$^3$H]TdR for 18 hr prior to harvest. To measure secondary in vitro responses to injected soluble antigens, chimeras were injected in the foot pad and at the base of the tail with 50 μg of KLH or FGG emulsified in CFA, and the draining LN cells were recovered 7 days later. Whole

LN cells (4–8 $\times$ $10^5$) were cultured with 10–20 $\mu$g/ml of the injected antigens for 5 days with [$^3$H]TdR (1 $\mu$Ci/well) added for the last 18 hr.

**Precursor Frequency Measurements**

To measure precursor frequency of reactive T cells using colchicine in bulk in vitro cultures (Wilson et al., 1968), 2 $\times$ $10^6$ LN cells from H2-M$^-$ or H2-M$^+$ mice were incubated with 5 $\times$ $10^6$ irradiated (2000 cGy) T-depleted stimulator spleen cells for 3 days in the presence of 1 $\mu$M colchicine and 5 $\mu$g/ml BrdU in 24-well plates under MLR conditions as previously described (Sprent and Schaefer, 1985). The cells were harvest at daily intervals and the frequency of BrdU$^+$ CD4$^+$ cells measured by FACS analysis after double staining for CD4 and BrdU. TCR V chain usage on the T cells responding to bm12 APCs was analyzed as follows. LN cells from the B6.PL→H2-M$^-$ chimeras were enriched for donor-derived CD4$^+$ cells by treating with a cocktail of MAbs to CD8, HSA, and Thy-1.2 plus C′. The cells were then depleted of CD44$^{hi}$ cells by staining with biotinylated anti-CD44 MAb followed by steptavidin-conjugated mini–magnetic beads and then passing through the magnetic columns (Mini-MACS, Miltenyi Biotec, Sunnyvale, CA). The enriched CD4$^+$ cells were incubated for 2 days with irradiated T-depleted bm12 spleen cells in the presence of 1 $\mu$M colchicine and then triple stained for TCR V chains, CD4, and CD69.

To measure precursor frequency using in vivo blood-to-lymph recirculation (Sprent et al., 1971; Atkins and Ford, 1975; Ford et al., 1975), young B6.Ly 5.1 (Ly-5.2$^-$, Thy-1.2$^+$) mice given 600 cGy were injected intravenously with a mixture of 4 $\times$ $10^7$ H2-M$^-$ (Thy-1.2$^+$, Ly 5.2$^+$) and 2 $\times$ $10^7$ B6.PL (Thy-1.2$^-$, Ly 5.2$^+$) LN cells. The spleen and the blood were harvested from the injected mice 24 hr later. PBLs, obtained by depleting red blood cells using hypotonic Tris ammonium acetate buffer, and spleen cells were triple stained for Ly 5.2, CD4, and Thy-1.2 and the percentage of Thy-1.2$^+$ cells analyzed on the Ly 5.2$^+$ CD4$^+$ cells. The proportion of reactive cells was determined as the percentage decrease in the ratio of H2-M$^-$/B6. PL CD4$^+$ cells in the blood compared to the injected mixture of cells.

**Depletion of Autoreactive T Cells by Thoracic Duct Cannulation**

H2-M$^-$ (Thy-1.2$^+$) LN cells (1 $\times$ $10^8$/host) were injected into irradiated (900 cGy) B6.PL (Thy-1.1$^+$) mice. The next day, thoracic duct cannulation was performed as previously described (Sprent, 1973). The lymph was collected at 20–40 hr after T cell injection; donor H2-M$^-$ CD4$^+$ cells were purified by treating the cells with a cocktail of anti-Thy-1.1, anti-CD8 and anti-HSA MAbs plus C′.

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