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The In Vivo Fate of APCs Displaying Minor H Antigen and/or MHC Differences Is Regulated by CTLs Specific for Immunodominant Class I-Associated Epitopes

Véronique Loyer, Pierre Fontaine, Stéphane Pion, Francis Hétu, Denis-Claude Roy, and Claude Perreault

The goal of this work was to evaluate the fate of APCs following interactions with T cells in unprimed mice with a normal T cell repertoire. We elaborated a model in which male adherent peritoneal mononuclear cells were injected into the foreleg footpads of naive female recipients mismatched for either minor or major histocompatibility Ags. At various times after injection, APC numbers in the draining (axillary and brachial) lymph nodes were assessed using a Ube1y gene-specific PCR assay. Our experimental model was designed so that the number of APCs expressing the priming epitope was similar to what is observed under real life conditions. Thus, early after injection, the frequency of afferent lymph-derived APCs expressing the priming epitope was in the range of 10^4–10^6 lymph node cells. We found that APCs presenting some, but not all, nonself epitopes were killed rapidly after entrance into the lymph nodes. Rapid elimination of APCs occurred following interactions with MHC class I-restricted, but not class II-restricted, T cells and was observed when APCs presented an immunodominant (B6*dom1/H7*), but not a nondominant (HY), epitope. Killing of APCs was mediated partly, but not exclusively, by perforin-dependent process. We propose that killing of APCs by CTLs specific for immunodominant MHC class I-restricted epitopes may be instrumental in regulating the intensity, duration, and diversity of T cell responses. The Journal of Immunology, 1999, 163: 6462–6467.

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It is generally considered that the duration of Ag presentation is limited to a large extent by the finite half-life of APCs. However, following in vivo interactions with T cells, it is not clear whether the demise of professional APCs is due to their basal turnover rate or to killing by Ag-specific T cells. It has been clearly demonstrated that under in vitro culture conditions, both CD4+ and CD8+ Ag-primed T cells kill dendritic cells, macrophages, and B cells in an Ag-specific manner (8, 9, 12–14). In vitro, killing of APCs by CD8+ CTLs is mediated primarily by the perforin pathway and to a lesser extent via FasL/Fas interactions, whereas CD4+ T cell cytotoxicity is conducted essentially by the Fas pathway (8, 9, 12–14). However, these studies have been performed with T cell lines or clones. Thus, although they show that Ag-primed T cells can rapidly eliminate APCs, they do not allow inferences regarding interactions of APCs with naive T cells. Furthermore, one caveat of in vitro studies is that they are performed under conditions quite distinct from those found in vivo, i.e., culturing a large excess of T cells in close proximity to APCs. Nevertheless, a few studies have addressed the fate of APCs following in vivo encounter with T cells. Thus, it has recently been reported that by 48 h after injection, OVA peptide-pulsed (but not unpulsed) DCs disappeared from the lymph nodes of mice bearing high numbers of TCR transgenic CD4+ T cells specific for an OVA peptide-I-A^d complex (15). Similarly, it has been shown that anti-HY TCR CD8^+ transgenic T cells could eliminate HY^+ B lymphocytes in vivo (16). These observations provide direct evidence that when present in relatively large numbers, CD4+ and CD8+ T cells from transgenic animals can kill APCs in vivo. However, the occurrence of this process in real life conditions and the tempo of APC demise have yet to be evaluated. Therefore, the goal of this work was to analyze the in vivo fate of hemopoietic APCs following the presentation of different T cell epitopes to naive nontransgenic animals with a normal T cell repertoire. These studies enabled evaluation of the half-life of APCs presenting specific class I- and/or
Materials and Methods

Mice
B10.C-H2ammer(H7) b (B10.H7) b mice were a gift from Dr. D. C. Roopenian (The Jackson Laboratory, Bar Harbor, ME). The following strains of mice were purchased from The Jackson Laboratory: BALB/c, B6.C-H2k/B10.C-H2ammer(H7) b (B10.H7), B6.C-H2ammer(H7)/Kheg (B12), B6.MRL-Fas b (B6Smn), B6Smn.C3H-Fasl b (gld), C3H.SW, C57BL/6 (B6), C57BL/6-Pp b (PKO), and C57BL/10J (B10). Mice used were between 6 and 16 wk of age and were maintained in specific pathogen-free conditions according to the standards of the Canadian committee for animal protection.

Preparation of cell suspensions and DNA extraction

Resident peritoneal mononuclear cells were harvested from euthanized male mice following injection of 10 ml of DMEM (Life Technologies, Gaithersburg, MD) supplemented with 2 mM glutamine and 5% FCS into the peritoneal cavity (17). These cell suspensions were enriched in mononuclear phagocytes by adherence to 25-cm2 tissue culture flasks (Sarstedt, Newton, NC) as previously described (18). Adherent peritoneal macrophages from male donors were gamma-irradiated (12.5 Gy), then 5 x 103 cells resuspended in 50 μl of DMEM were injected bilaterally into foreleg footpads of female recipients. Recipients were sacrificed at different times after injection, and their axillary and brachial lymph nodes were carefully dissected. DNA was extracted from lymph node cells using the Qiaamp Tissue Kit (Qiagen, Mississauga, Canada), following the manufacturer's protocol. The concentration and purity of the DNA samples were evaluated by spectrophotometry at 260 and 280 nm wavelengths.

Nested semiquantitative PCR amplification

Ube1y, a gene that maps to a region of the mouse Y chromosome required for normal spermatogonial proliferation (19, 20), was used as a marker gene to differentiate male from female cells. Conditions for PCR amplification were optimized using serial dilutions of B6 male cells admixed with B6 female cells so that one male cell in 1 x 106 female cells was reproducibly detectable. For each sample, PCR amplification was performed on six aliquots of 1 μg of purified DNA. For the first amplification, each DNA aliquot was amplified in a 50-μl reaction volume containing 25 pmol of each oligonucleotide primer, 0.2 mM dNTPs, 2.5 U of Taq DNA polymerase (Life Technologies), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 1.5 mM MgCl2. Oligonucleotides for this first amplification of the DNA samples were 5′-TGTGGTGCGCTTGCGACGCTAGACT-3′ and 5′-TCAA CAAAAGGCCTCTCTTC-3′, and generated a 525-bp fragment. DNA was subjected to amplification in a Gene Amp PCR system 9600 (PE Applied Biosystems, Foster City, CA). Cycle conditions included an initial 94°C denaturation step of 4.5 min followed by 25 cycles consisting of a 94°C denaturation step (30 s), a 55°C annealing step (15 s), and a 72°C extension step (15 s). The final extension time was prolonged to 5 min.

Upon completion of the first amplification round, 5 μl of the amplified mixture was reamplified for another 25 cycles using the same parameters as those described above, except for the use of nested oligonucleotide primers. For this second amplification, oligonucleotide primers were internal to the original primers (5′-GACATTTCCTCGGAGAAAAG-3′ and 5′-CATGTCATTAGACAGGAGCAG-3′) and generated a smaller 377-bp fragment.

Aliquots of 10 μl from the second PCR amplification were analyzed by electrophoresis in 1% agarose gel (Bio-Rad Laboratories, Hercules, CA) in Tris-acetate-EDTA buffer containing 0.5 μg/ml of ethidium bromide and were visualized under UV light. The first PCR amplification product of positive samples was then serially diluted and reamplified under the same conditions as those described above for the second PCR amplification. The number of dilutions generating a UV-detectable signal permitted quantification of the samples (21). In each experiment, a positive control consisted of B6 male cells, and a negative control consisted of B6 female cells. PCR procedures included strict measures to avoid contamination (22, 23).

Results

Experimental protocol

We developed our experimental protocol in an effort to mimic real life conditions. Thus, we elected to measure the survival of realistic numbers of APCs carried by the afferent lymph into peripheral lymph nodes. Careful studies during the course of respiratory Sendai virus infection in mice have shown that frequencies of APCs presenting MHC class I- and MHC class II-restricted viral epitopes reach a peak of ~104/106 lymph node cells (24). Detection of such low cell frequencies is best accomplished by PCR methodology. Therefore, we elaborated a model in which male APCs are injected into the foreleg footpads of female recipients, and, at various times after injection, the numbers of male APCs in the draining axillary and brachial lymph nodes were measured using a previously validated sensitive semiquantitative nested PCR assay (21). We used Ube1y, a Y-linked gene, as a marker gene. To estimate the frequency of male APCs, DNA extracted from recipient's axillary and brachial lymph nodes was amplified, and the product of this first amplification reaction was serially diluted 10-fold from 105 to 10−3. Each fraction was PCR amplified with nested oligonucleotide primers, then aliquots of the second amplification products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. The results depicted in Fig. 1A show that this assay was reproducible (cf., concordance between samples), specific (female cells always gave negative results), and sensitive, as it could detect DNA from one male cell in 106 female cells. Furthermore, within the range tested, there was a log-linear relationship between the number of PCR-positive bands and the number of male cells in the sample. Thus, this methodology provided a good estimate of the proportion of male cells over the range of cell frequencies of interest for this study.

We used adherent peritoneal mononuclear cells as a source of APCs. Such preparations contain ~66% monocytes/macrophages,
33% B lymphocytes, and 1% DCs (17, 18, 25, 26). Peritoneal cells were irradiated (12.5 Gy) to preclude proliferation into allogeneic recipients. In dose-finding experiments, we observed that 2–24 h after injection of 5 × 10^5 male APCs into each foreleg footpad of female B6 recipients, we reproducibly obtained the desired APC frequency in axillary and brachial lymph nodes, i.e., 10^4–10^5 APCs/10^6 lymph node cells (Fig. 1). When assessed at various time points, no male cells could be detected outside the draining lymph nodes, i.e., in spleen or in inguinal lymph nodes (data not shown). Female mice with the H2b haplotype, such as B6, can generate T cell responses toward HY epitopes encoded by the Y chromosome. Conversely, other strains of mice, such as BALB/c, are totally unresponsive to HY epitopes (27). To verify whether HY-specific responses could influence APC recovery under our assay conditions, we compared the recoveries of male B6 and BALB/c APCs 24 and 96 h after injection into syngeneic female recipients. No difference was observed between B6 and BALB/c mice (Fig. 1), showing that the presence of HY epitopes does not influence the short term recovery of injected APCs.

**The in vivo fate of APCs presenting various nonself epitopes**

Mice were sacrificed between days 1 and 21 after s.c. injection of APCs, and the frequency of (male) APCs in their axillary and brachial lymph nodes was assessed by PCR assay. The survival of injected cells was estimated in a variety of mouse strain combinations from 1 to 21 days following injection. Correlation between the number of positive PCR dilutions and the frequencies of male APCs is shown in Fig. 1A. Each bar represents one mouse.

![PDF Image](http://www.jimmunol.org/)

**FIGURE 2.** The in vivo fate of APCs presenting mismatched minor and/or major histocompatibility Ags. A–G depict the PCR-positive dilutions of male APCs recovered in the lymph nodes (axillary and brachial) of female recipients in seven donor/recipient combinations from 1 to 21 days following injection. Correlation between the number of positive PCR dilutions and the frequencies of male APCs is shown in Fig. 1A. Each bar represents one mouse.

<table>
<thead>
<tr>
<th>Injected (Male)</th>
<th>Recipients</th>
<th>Target Epitope(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>B6</td>
<td>HY</td>
</tr>
<tr>
<td>B10</td>
<td>B10.C-H7b</td>
<td>HY + H7a (B6 bm1)</td>
</tr>
<tr>
<td>B6</td>
<td>C3H/He</td>
<td>MHC class I + class II (H-2b)</td>
</tr>
<tr>
<td>C3H/He</td>
<td>B6</td>
<td>MHC class I + class II (H-2b)</td>
</tr>
<tr>
<td>bm1</td>
<td>B6</td>
<td>HY + MHC class I (K bm1)</td>
</tr>
<tr>
<td>bm12</td>
<td>B6</td>
<td>HY + MHC class II (I-A bm12)</td>
</tr>
<tr>
<td>bm1</td>
<td>gld</td>
<td>HY + MHC class I (K bm1)</td>
</tr>
<tr>
<td>bm1</td>
<td>PKO</td>
<td>HY + MHC class I (K bm1)</td>
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The time required for the number of APCs to decrease below a threshold of 10 APCs/10^6 lymph node cells (i.e., PCR negative at a 10^-2 fold dilution) was taken as a useful criterion to compare APC survival in selected donor/recipient combinations. As shown in Fig. 2, the nature of nonself Ag expressed by APCs had a dramatic influence on their survival. Thus, at one end of the scale, HY had little, if any, influence on the persistence of APC, because only on day 21 did the frequency of APCs drop below the threshold of 10 cells/million in one of two mice tested (Fig. 2A). In contrast, APCs incompatible for a full MHC haplotype (H2b or H2a) were rapidly eliminated as their frequency dropped below this threshold 2–3 days after injection (Fig. 2, B and C).

MHC class I (bm1) and class II (bm12) mutants differ by only a few amino acids from the wild-type molecules, and they elicit pure CD8+ and CD4+ T cell responses, respectively (28–30). Therefore, they represent a most useful paradigm to study MHC class I- and class II-restricted responses. Interestingly, MHC class I and class II incompatibilities had quite divergent effects on the persistence of APCs. Class I incompatibility (bm1→B6; Fig. 2F) was sufficient to induce APC disappearance as rapidly as that seen in the case of a full MHC mismatch. In contrast, class II incompatibility (bm12→B6; Fig. 2G) had no apparent influence on APC survival. Similar results were observed in the reciprocal combinations, i.e., B6→bm1 and B6→bm12 (data not shown). These results indicate that induction of class I-restricted, but not class II-restricted, T cell responses can lead to rapid clearance of APCs.

Interpretation of the above results must take into account the fact that, like their wild-type homologs, MHC mutant molecules are expressed at ~10^3 copies/APC, so that their cell surface abundance is superior to that of most conventional epitopes, which are usually expressed at ~10^3 copies/cell (31, 32). Thus, the question arises as to whether more typical class I-restricted T cell epitopes, with a cell surface density on the order of 10^3–10^4 copies/cell, can cause rapid demise of APCs. We have shown above that a single HY disparity had little or no effect on APC clearance (Fig. 2A). However, the single class I-restricted HY peptide presented by H2b mice is expressed at only 10 copies/cell and is a nondominant epitope, i.e., it can elicit a T cell response when presented alone, but is neglected when presented together with other epitopes on the same APCs (33, 34). Conversely, the B6 bm1 MiHA, a nonapeptide presented by the H2-D9 molecule, is expressed at 10^4 copies/cell and is immunodominant when presented with numerous other MHIAs (33–35). It has recently been found that B6 bm1 is encoded by the H7 MiHA locus originally discovered by Snell 35 years ago (36, 37). Thus, by injecting B10 APCs into H7 congenic recipients, we could evaluate the impact of the immunodominant B6 bm1 epitope (encoded by the H7a allele) on APC clearance. In
this strain combination, APC frequency decreased below 10 cells/million by day 8 (Fig. 2E). Similar results with regard to the kinetics of APC clearance were observed in the B6→C3H.SW strain combination (Fig. 2D) as well as in the reciprocal combination C3H.SW→B6 (data not shown), in which APCs present multiple incompatible MiHAs. These observations demonstrate that presentation of immunodominant epitopes such as B6<sup>dom1</sup> (H7<sup>a</sup>), either alone (B10→B10.C-H7<sup>br</sup>) or together with other Ags (B6→C3H.SW), leads to a rapid clearance of APCs. Considering that bm1 mutants probably elicit more vigorous CD8<sup>+</sup> T cell responses than most conventional microbial epitopes, the clearance of B6<sup>dom1</sup>-incompatible APCs may be more representative of what can be expected with conventional Ags under real life conditions.

**Clearance of APCs is mediated partly, but not solely, by the perforin pathway**

In an effort to gain some insight into the mechanisms responsible for the clearance of APCs, we studied the fate of APCs in strain combinations in which the perforin- or Fas-dependent cytocidal pathway was defective (Fig. 3). Taking into account the finding that clearance of APCs was regulated essentially by MHC class I-restricted effectors (Fig. 2), we followed the fate of bm1 APCs after injection into perforin- or FasL-deficient B6 recipients. Whereas bm1 APCs dropped below the threshold of 10 cells/million by day 2 following injection into normal (Fig. 2F) or FasL-deficient hosts (Fig. 3A), they persisted for 4–14 days when injected into perforin-deficient hosts (Fig. 3B). These observations provide evidence for a role of perforin, but not Fas, in the clearance of APCs. It is notable, however, that the APC clearance rate was substantially more variable (4–14 days) in perforin-deficient recipients than in other hosts. This suggests that although they may be less effective, perforin-independent pathways can contribute to the clearance of APCs.

**Discussion**

Three conclusions can be drawn from this study. First, APCs presenting some, but not all, nonself epitopes can be killed rapidly after entrance into the lymph nodes of normal mice with a normal naive (preimmune) T cell repertoire. Second, elimination of APCs requires MHC class I-restricted interactions with T cells and is observed when APCs present an immunodominant (B6<sup>dom1</sup>/H7<sup>a</sup>), but not a nondominant (HY), epitope. Finally, clearance of APCs is mediated partly, but not exclusively, by the perforin pathway. Naturally, as our studies were limited to the first 3 wk following APC injection, we cannot discard the possibility that class II and/or nondominant epitopes could have more subtle effects on APC clearance detectable only at later time points. Furthermore, as this work was based on evaluation of peritoneal APCs, i.e., essentially a mixture of monocytes/macrophages and B lymphocytes, it will be worthwhile in future studies to evaluate whether purified preparations of macrophages, B cells, and DCs share the same behavior.

Our conclusions regarding the differential effects of class I- vs class II-restricted interactions are based on the study of bm1 and bm12 mutants. Studies regarding in vitro and in vivo T cell proliferation as well as induction of graft-vs-host disease and skin graft rejection have shown that responses of B6 T cells to bm1 and bm12 Ags have similar kinetics (time of onset and expansion rate) and intensity (29, 38, 39); responses against bm1 are neither more rapid nor more vigorous. Thus, the rapid clearance of bm1, but not bm12, APCs reported herein (Fig. 2) cannot be ascribed to a superior immunogenicity of bm1 relative to bm12. Rather, under the conditions we employed, this finding provides strong evidence that interactions with CD4<sup>+</sup> and CD8<sup>+</sup> T cells have qualitatively different consequences for the APC, and that only class I-restricted interactions are lethal for the APC. Therefore, it appears logical to infer that one important function of class I-restricted CTLs may be to control the duration of Ag presentation. Furthermore, selective killing of APCs by CD8<sup>+</sup> T cells could explain previous documentation that, at least in vitro, expansion of anti-bm1 CD8<sup>+</sup> T cells is of shorter duration than that of anti-bm12 CD4<sup>+</sup> T cells (29).

The delayed clearance of APCs in perforin-deficient hosts indicates that the perforin pathway is one, but not the sole, pathway that regulates the fate of APCs. The influence of perforin is consistent with observations showing that under most circumstances, MHC class I-restricted CTLs lyse target cells preferentially via the perforin pathway (40, 41). However, the results presented in Fig. 3 also indicate that APC demise can be induced by perforin-independent mechanisms, and that Fas has a minor influence, if any, on the fate of APCs. It has been shown that effector CTLs harbor potent killing mechanisms in addition to those provided by the Fas and perforin/granzyme B pathways (42). These alternative pathways involve members of the TNF family, ATP, IFN-γ, as well as some undefined signal elicited by TCR binding of the α2 domain of MHC class I molecules on the target cell (43–47). A plausible rationale for this redundancy of lethal mechanisms is to deal with the array of antiapoptotic molecules elaborated by intracellular pathogens to extend the life of infected cells (48). Clearly, further studies will be required to decipher the mechanisms responsible for the perforin-independent clearance of APCs shortly after productive interactions with CTL precursors. It is noteworthy that the persistence of APCs in PKO recipients (4–14 days; see Fig. 3B) was substantially more variable than that in other mice. This suggests that the efficiency of the other mechanisms may be less predictable than that of the perforin pathway.

The differential influence of dominant vs nondominant epitopes on APC survival impinges on our understanding of the mechanisms of immunodominance. MHC class I-restricted immunodominant epitopes differ from nondominant epitopes in two ways (49). First, when both types of Ag are presented separately, dominant epitopes evoke more powerful T cell responses (34, 50–52). Second, when dominant and nondominant epitopes are presented together, recognition of the dominant epitope suppresses responses to nondominant epitopes, and thereby restricts the diversity of the repertoire of CTL responses (33, 53). This inhibitory effect of dominant epitopes on recognition of nondominant epitopes has been referred to as the immunodominance effect or immunodomination and is a central feature of all CTL responses that follow
confrontation with numerous epitopes (54, 55). Interestingly, immunodominance is observed only when both the dominant and nondominant epitopes are presented at the same time on the same APC (33, 49). Suppression of CTL responses to nondominant epitopes does not take place when the dominant and nondominant epitopes are presented at different time points or if they are presented concomitantly but on separate APCs. The present demonstration that APC survival is curtailed following presentation of an immunodominant (B6dom1), but not a nondominant (HY), class I-restricted epitope could provide a plausible basis for immunodominance. Accordingly, we propose that by killing APCs, CTLs specific for immunodominant determinants reduce the duration of Ag presentation and consequently impede the generation of CTL responses toward nondominant Ags. It is notable that, like APC killing, immunodominance seems to be primarily a characteristic of CD8+ as opposed to CD4+ T cell responses (49, 56).

Conceptually, it is tempting to speculate that killing of APCs by CTLs specific for immunodominant epitopes may represent a sensible way to regulate T cell responses. 1) It would terminate Ag presentation only when CTLs specific for the best (immunodominant) epitopes have been primed (49). 2) In the case of infection with many intracellular micro-organisms, killing of monocyte-macrophage APCs would lead to elimination of an important reservoir of pathogens. 3) Limiting the duration of Ag presentation may reduce the risk of T cell exhaustion, and thus of tolerance. 4) Restricting the diversity of the T cell repertoire could lessen the risk of collateral autoimmune damage by cross-reactive CTLs (54, 57). Finally, it makes good sense that in a system where regulation of immune responses involves killing APCs, Ag presentation be conducted primarily by disposable cells with a rapid renewal rate, such as professional hemopoietic APCs.

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