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Alloreactive T Cells That Do Not Require TCR and CD8 Coengagement Are Present in Naive Mice and Contribute to Graft Rejection

Pamela A. Smith*† and Terry A. Potter2*†

Class I alloreactive CTL populations have been defined as either CD8 dependent or CD8 independent, based upon their ability to kill target cells in the presence of Ab to CD8. The CD8-dependent population uses CD8 in a coreceptor role with the TCR, and mutations in the class I molecule that destroy the CD8 binding site abrogate CTL killing, even if the target cell expresses other allelic forms of class I molecules with an intact binding site for CD8. The CD8-independent population apparently does not require CD8, as Ab to CD8 has no effect on the ability of these cells to kill appropriate target cells. We have isolated a third population of CTL that is inhibited by the addition of CD8 Ab yet can kill target cells that express the alloantigenic molecule incapable of binding CD8, provided that the target cells also express non antigenic class I molecules that contain an intact binding site for CD8. We refer to these cells as CD8 bystander-dependent CTL. Many (10 of 12) of these CTL were able to kill H-2Kb-expressing transfectants of T2 cells, consistent with the idea that they recognize a peptide-independent determinant that may be expressed at a high density on the cell surface. These CD8 bystander-dependent CTL are only readily detectable in vitro when spleen cells from mice primed in vivo with a skin graft are used.

The term accessory molecule has been used to describe the activities of CD4 and CD8 when they are unable to bind the same MHC molecule as the TCR. There are conflicting data as to whether the binding of MHC molecules in an accessory manner contributes to T cell activation. While some studies demonstrated that the effects mediated by CD4 or CD8 as accessory molecules do not contribute to T cell activation (9–12), several T cell hybridomas have been derived in which an accessory role for CD4 or CD8 enhances responsiveness (13–16). In addition, other studies have shown that following Ab-mediated ligation of the TCR, the binding of CD8 to nonantigenic, or bystander, class I molecules leads to T cell activation, as measured by serine esterase release and hydrolysis or phosphatidylinositides (9–11). What is unclear in this system is whether the Ab-mediated ligation of the TCR has effects equivalent to those of the physiologic signaling through TCR only or, because of the high degree of TCR occupancy, the signaling events initiated are similar to those that occur upon TCR and CD8 coengagement. In a previous study we found that in CD8-dependent CTL, TCR and CD8 coengagement is required for phosphatidylinositide hydrolysis, whereas in CD8-independent CTL, engagement of the TCR is sufficient for killing, but not for phosphatidylinositol hydrolysis (12). Furthermore, in these CD8 independent CTL, which seemingly do not require CD8 to stabilize the binding of the TCR to the MHC molecule, CD8 and TCR engagement by distinct MHC class I molecules is capable of activating phosphatidylinositol hydrolysis (12). In this report we have further examined the contribution of CD8 to T cell activation under conditions where it is unable to function as a coreceptor.

Most of the previous studies on the coreceptor vs accessory molecule function of CD8 and CD4 have focused on their roles in isolated effector cells. There are, however, a number of investigations that demonstrate differences in the requirements for stimulation of naive or activated T cells. We had noted previously that in the in vitro allo-response of naive T cells to cells expressing the α3 domain mutant molecules was very weak (6), implying a requirement for CD8 coreceptor function for the activation of naive T cells. We therefore have investigated the fate of a skin graft in
which the only incompatibility is the expression on the donor graft of a mutant class I molecule incapable of binding CD8. We describe herein that such grafts are rapidly rejected. One of the components of this alloreponse is CD8\(^+\) class I reactive CTL, which, for cytotoxicity and secretion of IFN-\(\gamma\), require CD8 engagement, although not necessarily coengagement with the same MHC class I molecule as the TCR. This population of alloreactive CTL, which we term CD8 bystander dependent, can be distinguished from the classically described CD8-dependent and CD8-independent CTL populations. These CTL represent a distinct subset of CD8-dependent CTL, as they require the binding of CD8; however, unlike most class I-restricted T cells, it is sufficient for CD8 to function as an accessory molecule and not necessarily as a coreceptor. Furthermore, this CTL population is not unique to stimulation with the mutant class I molecule and can also be isolated following stimulation with the wild-type molecule.

Materials and Methods

Mice

B10.D2, BALB/c, C57BL/6, and SJL mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were bred to produce (BALB/c \(\times\) B10.D2) F\(_1\) (referred to herein as CD2F\(_1\)) and (B6 \(\times\) SJL) F\(_1\) progeny. 28.1 mice are transgenic for the H-2\(K^b\) gene on a B10.D2 background (8). B6D8 mice are transgenic for the wild-type H-2\(D^d\) gene on a C57BL/6 background (13). 29.2 mice are transgenic for the mutant H-2\(D^d\) gene (non-CD8 binding) on a C57BL/6 background.

Target cell lines

M12.C3 (H-2\(\text{b}\)) (14), the peptide-processing defective cell line, T2 (15–19), and the nonmutant parental cells from which T2 were derived, T1 cells (15), expressing the wild-type (H-2\(K^b\)-wt) or non-CD8 binding a3 domain mutant (H-2\(K^b\)-m) H-2\(K^b\) class I molecules, were generated by transfection. These cells are referred to herein as M12-, T2-, or T1-K\(_b\) wild-type (or K\(_b\)-wt) or as M12-, T2-, or T1-K\(_b\)-mutant (or K\(_b\)-m). RMA/S (20–22) cells are a peptide-processing defective line of the H-2\(\text{a}\) genotype.

Generation of T cell clones

All T cell clones described were isolated by limiting dilution from the spleens and lymph nodes of CD2F\(_1\) mice that had been engrafted with trunk skin from a 29.7 strain transgenic mouse. In addition, several T cell lines were isolated by limiting dilution from the spleens and lymph nodes of CD2F\(_1\) mice that had been engrafted with trunk skin from a 28.1 strain transgenic mouse. Lymph node and spleen cells were harvested on day 14 following engraftment and stimulated in vitro with irradiated 29.7 (clones) or 28.1 (lines) spleen cells. The clones were also restimulated weekly with irradiated 29.7 stimulator spleen cells. Responder and stimulator cells were cocultured in DMEM/FCS (10%) containing Con A-stimulated rat spleen supernatant (T cell medium). All clones characterized in this study were confirmed, by flow microfluorometric analysis, to be CD8\(^+\)/a\(\beta\) T cells (data not shown). The alloreactive H-2\(K^b\) CTL clone 4.1 was generated by limiting dilution cloning from a primary MLR of B10.D2 stimulated with C57BL/6 spleen cells.

Assay of cytotoxic reactivity

Cytotoxic activity was measured in a standard \(^{51}\)Cr release assay. Target cells were labeled with \(^{51}\)Cr (100 \(\mu\)Ci/10\(^6\) cells) for 1.5 h at 37°C. Labeled target cells (5000/well) were added to each well of a V-bottom microtiter plate and incubated with effectors at the indicated E:T ratios at 37°C for 4 to 6 h. The percent specific release was calculated using the following formula: \(\left(\frac{E - S}{M - S}\right) \times 100\), where \(E\) is the average experimental release of duplicate samples, \(S\) is the average spontaneous release of triplicate samples, and \(M\) is the average maximum release of triplicate samples. Spontaneous release was measured in the absence of effector cells, and maximum release was measured in the presence of 1% Nonidet P-40 instead of effector cells.

Preparation of peptide extract

Cell suspensions prepared from the spleens of C57BL/6 mice were washed extensively and then homogenized in 0.1% trifluoroacetic acid. The suspension was adjusted to pH 2.0 with HCl, sonicated 20 times for 1 s each time, and then incubated at 4°C for 60 min. Cellular debris was removed by centrifugation at 2000 \(\times\) g for 20 min, and the supernatant was passed through a Centricon 10 membrane, lyophilized, and resuspended in sterile distilled water.

Precursor frequency analysis

Spleen cells from CD2F\(_1\) mice were cultured at titrated concentrations, each concentration repeated 24 times, in V-bottom 96-well plates. The CD2F\(_1\) mice were either unprimed or primed with an H-2\(K^b\)-incompatible skin graft. The engrafted mice were sacrificed 2 wk following engraftment, a period sufficient in all cases for complete graft rejection. CD2F\(_1\) cells were stimulated with \(^{10}\) irradiated spleen cells/well and, in addition, \(^{10}\) irradiated spleen cells from the responder. All cultures included 10% rat spleen cell Con A supernatant as a source of IL-2. Following 1 wk of stimulation, the cultures were assayed for cytolyis of \(^{51}\)Cr-labeled target cells during a 4-h incubation. The precursor frequency was calculated by determining the cell concentration at which 37% of the wells were negative, using a graph of the log percent negative wells on the ordinate and the responder cell concentration on the abscissa. Determination of the cutoff for positive wells was made by culturing 24 wells of responder cells at the highest concentration used in a particular assay with no stimulators, and then calculating the average CTL value readout plus 3 SDs.

Results

Skin grafting analysis

CD2F\(_1\) mice were engrafted with trunk skin from either 28.1 (K\(_b\)-wt) or 29.7 (K\(_b\)-m) mice. The grafts were monitored daily, and rejection was determined by complete graft ablation. Depletion of T cells in vivo was accomplished by injection of Abs 2 wk following thymectomy. Mice were thymectomized at 3 to 4 wk of age and 2 wk later were injected i.p. three times (at 3-day intervals) with 200 \(\mu\)l of ascites fluid containing either CD4 (GK 1.5) or CD8 (YTS 169) Ab. The effectiveness of the Ab-mediated depletion was monitored with biotin-conjugated Abs to CD4 or CD8 that reacted with an epitope distinct from the depleting Ab (data not shown).

Skin grafts that express a disparate class I molecule that does not engage CD8 are rejected as rapidly as grafts that express wild-type class I

To examine the requirement for TCR and CD8 coengagement in skin graft rejection, CD2F\(_1\) (H-2\(b\)) mice were engrafted with H-2\(K^b\)-wt, or H-2\(K^b\)-m-incompatible skin. Skin grafts expressing either form of H-2\(K^b\) are rejected with similar kinetics (Table I). Rejection is T cell mediated, because depletion of both CD4\(^+\) and CD8\(^+\) in thymectomized recipient results in indefinite (120+ days) acceptance of the graft. The 28.1 transgenic mouse was initially derived in (C57BL/6 \(\times\) BALB/c) F\(_2\) fertilized eggs and, despite repeated backcrossing, could theoretically still retain some minor histocompatibility genes derived from BALB/c. By using CD2F\(_1\) mice as recipients we have avoided the possibility that the rejection is directed at any BALB/c histocompatibility genes retained by the 28.1 mouse. One possibility for the vigorous response against the mutant H-2\(K^b\) molecule, which is not observed
CD8 Bystander-Dependent CTL

Table I. Rejection of wild-type and mutant H-2Kb incompatible skin grafts

<table>
<thead>
<tr>
<th>Graft Recipient</th>
<th>Graft Donor</th>
<th>Days for Rejection (Mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2F1</td>
<td>28.1 (Kbwt)</td>
<td>13.7</td>
</tr>
<tr>
<td>CD2F1</td>
<td>29.7 (Kbm)</td>
<td>12.5</td>
</tr>
<tr>
<td>CD2F1, (CD4 and CD8 depleted)</td>
<td>28.1 (Kbw)</td>
<td>120+</td>
</tr>
<tr>
<td>CD2F1, (CD4 and CD8 depleted)</td>
<td>29.7 (Kbm)</td>
<td>120+</td>
</tr>
<tr>
<td>28.1 (Kbw)</td>
<td>29.7 (Kbm)</td>
<td>120+</td>
</tr>
<tr>
<td>(B6 × SJL)F1</td>
<td>B6D8 (Ddwt)</td>
<td>16.1</td>
</tr>
<tr>
<td>(B6 × SJL)F1</td>
<td>29.2 (Ddwt)</td>
<td>16.9</td>
</tr>
<tr>
<td>B6D8 (Ddwt)</td>
<td>29.2 (Ddwt)</td>
<td>120+</td>
</tr>
</tbody>
</table>

*Skin grafts were performed and monitored as described in Materials and Methods. 28.1 (Kbw) and 29.7 (Kbm) refer to the B10.D2 H-2Kbw and H-2Kbm transgenic mice, respectively. B6D8 (Ddwt) and 29.2 (Ddwt) denote the C57BL/6 H-2Ddwt and C57BL/6 H-2Ddwt transgenic mice, respectively.

in vitro, is that the H-2Kbm molecule is the source of a unique peptide not present in the H-2Kbw molecule. When presented by an H-2d-encoded molecule, this theoretical peptide could constitute the alloreactive component upon which the rejection response is based. To address this possibility, several 28.1 (Kbw) and 29.7 (Kbm) mice were grafted with skin from 29.7 (B10.D2 H-2Kbm) mice. The grafts showed no sign of rejection for 120+ days, indicating that there is no peptide derived from H-2Kbm that is presented by H-2d to provide an allodeterminant.

To confirm that rapid skin graft rejection across a single MHC class I molecule incompatibility in the absence of CD8/MHC coengagement is not unique to H-2Kb, we performed similar grafts in which the only disparity was the expression of H-2Dd mutant class I molecules incapable of binding CD8. These grafts were rejected as rapidly as those in which the disparity was a wild-type H-2Dd molecule. Cells that express the H-2Dd molecule is incapable of initial engagement of CD8, therefore, in contrast to CD8-independent CTL, CD8 bystander-dependent CTL are inhibited by Ab to CD8 (see below). This dependence on CD8 for killing of M12.Kbm cells, in which the H-2Kb molecule is incapable of initial engagement of CD8, strongly suggests that the CD8 molecule is binding to the H-2d-encoded class I molecules.

We selected two (designated clones 15 and 28) of the 10 CD8 bystander-dependent CTL clones that could kill T2.Kbw cells for further analysis. We also included the two clones (no. 5 and 10) levels of the human HLA molecules (23). Therefore, T2 cells transfected with murine class I MHC genes, such as H-2Kbw or H-2Kbm, express these molecules either without peptide, or in association with a limited array of peptides (24). Furthermore, the human class I molecules that are expressed do not significantly engage murine CD8 (25–27); consequently, class I molecules expressed by the transfected gene are the only possible target for the binding of murine CD8. We have previously described several other CTL clones that were determined to be CD8 independent, as they were able to lyse T2.Kbm cells and were unaffected by the addition of CD8 Ab (28). In contrast to the previously described CTL clones, which could kill both T2.Kbw and T2.Kbm cells, 10 of the 12 clones represented in Figure 1 were able to kill T2.Kbw cells, but none were able to kill T2.Kbm cells. These 12 CTL clones that were able to recognize M12.Kbm, but not T2.Kbw, cells are considered to be peptide independent, whereas the two clones that were unable to kill T2.Kbm cells were considered to be peptide dependent. The CTL that we had previously characterized as able to kill T2.Kb cells were shown to be peptide independent based on several criteria, including the ability to kill T2.Kb cells treated with acid to remove any MHC-bound peptide and the ability to respond to plate-bound H-2Kb produced by transfected Drosophila melanogaster cells (28). In the current study we did not perform such exhaustive analysis to establish the peptide-independent nature of the recognition by the 10 clones that killed T2.Kbw; thus, we cannot exclude the possibility that these CTL are, in fact, specific for one of the limited array of peptides expressed in association with H-2Kb on T2.Kb cells. Cells that express the α3 domain mutation are also killed by CD8-independent CTL (28); however, in contrast to CD8-independent CTL, CD8 bystander-dependent CTL are inhibited by Ab to CD8 (see below). This dependence on CD8 for killing of M12.Kbm cells, in which the H-2Kb molecule is incapable of initial engagement of CD8, strongly suggests that the CD8 molecule is binding to the H-2d-encoded class I molecules.

FIGURE 1. Cytolysis of M12.Kbw, M12.Kbm, T2.Kbw, and T2.Kbm target cells by 12 clones isolated from a CD2F1 mouse following rejection of a skin graft expressing H-2Kbm. Lysis at a 10:1 E:T cell ratio for an individual clone is represented by a symbol. These T cell clones cannot kill target cells that express only the H-2Kb class I molecule with the α3 domain mutation (i.e., T2.Kbm), but can kill cells that express other nonantigenic class I molecules in addition to the mutated H-2Kb (i.e., M12.Kbm). Also shown is that two of these clones (no. 5 and 10) do not kill T2.Kbw cells.
that killed M12.Kb\textsuperscript{m}, but not T2.Kb\textsuperscript{wt}, cells. Addition of an extract from C57BL/6 spleen cells that contains MHC binding peptides sensitized T2.Kb\textsuperscript{wt}, but not T2.Kb\textsuperscript{m}, cells for lysis by clones 5 and 10 (Fig. 2). This observation together with their ability to kill T1.Kb\textsuperscript{wt} cells (data not shown) suggests that clones 5 and 10 are peptide dependent and CD8 dependent. To confirm the specificity of these CD8 bystander CTL clones, we analyzed the abilities of the four clones (no. 5, 10, 15, and 28) to kill M12.C3 cells transfected with the H-2K\textsuperscript{b} genes. All four clones killed both M12.Kb\textsuperscript{wt} and M12.Kb\textsuperscript{m} cells, but did not kill nontransfected M12.C3 (H-2\textsuperscript{d}) cells (Fig. 3), indicating that these clones are indeed specific for H-2K\textsuperscript{b}. The CD8 coreceptor-dependent CTL clone 4.1, which cannot kill M12.Kb\textsuperscript{m} cells, was included in these experiments for comparative purposes.

In addition to testing the ability of CD8 bystander-dependent clones to kill target cells that express the mutant or wild-type H-2K\textsuperscript{b} molecule, we determined whether the CD8\textalpha Ab (YTS.169) would inhibit killing by the four CD8 bystander-dependent CTL clones. The addition of YTS.169 completely inhibited the lysis of M12.Kb\textsuperscript{wt} and M12.Kb\textsuperscript{m} cells by the two peptide-independent clones (no. 15 and 28; Fig. 4) as well as by the peptide-dependent CTL clones (no. 5 and 10; data not shown). The killing of T2.Kb\textsuperscript{wt} cells by the peptide-independent CTL clones (no. 15 and 28) was also inhibited by addition of CD8 Ab (Fig. 4). The ability of CD8 Ab to inhibit cytolysis by these clones supports the hypothesis that CD8 bystander T cells require CD8 engagement to perform cytolysis; however, as demonstrated by its ability to kill M12.Kb\textsuperscript{m} cells, the class I molecule to which CD8 binds may be distinct from the class I molecule that interacts with the TCR.

**Figure 2.** Cytolysis of T2, T2.Kb\textsuperscript{wt}, and T2.Kb\textsuperscript{m} target cells by CD8 bystander-dependent CTL clones 5 (denoted by squares) and 10 (circles). These CTL assays were conducted in the absence (top) or the presence (bottom) of an extract from C57BL/6 spleen cells that contains MHC binding peptides. These clones are cytolytic for T2.Kb\textsuperscript{wt} cells only when pulsed with the extract from C57BL/6 spleen cells, suggesting that they are peptide dependent. These clones cannot, however, kill T2.Kb\textsuperscript{m} cells when pulsed with the C57BL/6 peptide extract, indicating that they require CD8 engagement.

**Figure 3.** Cytolysis by the CD8 bystander-dependent CTL clones (open squares indicate clone 15; closed circles indicate clone 5) and the CD8-dependent CTL clone 4.1 (closed triangles) of M12.C3 (H-2\textsuperscript{a}), M12.Kb\textsuperscript{wt}, and M12.Kb\textsuperscript{m} target cells. The other two bystander-dependent clones, 10 and 28, were indistinguishable from clones 15 and 5 and are therefore not included in the figure. Failure to kill M12.C3 cells demonstrates specificity for H-2K\textsuperscript{b}.

**T cells that are CD8 bystander dependent for lysis are also CD8 bystander dependent for IFN-\gamma release**

We assayed IFN-\gamma secretion by the CD8 bystander-dependent CTL clones upon incubation with M12.Kb\textsuperscript{b} and T1.Kb\textsuperscript{b} cells. T1.Kb\textsuperscript{b} cells were used instead of T2.Kb\textsuperscript{b} cells so that the IFN-\gamma responses of both the peptide-dependent and the peptide-independent clones could be determined. The clones secreted IFN-\gamma upon incubation with M12.Kb\textsuperscript{m} cells, but did not produce IFN-\gamma upon incubation with T1.Kb\textsuperscript{m} cells (Fig. 5). The difference in the response to these two cell lines suggests that the clones require CD8 engagement for IFN-\gamma secretion, and furthermore, that CD8 engagement of non-antigenic (i.e., a different allelic form that engaged by the TCR) MHC class I molecules is sufficient for IFN-\gamma release. For comparative analysis, the CTL clone, 4.1, that is CD8 coreceptor dependent for killing and for IFN-\gamma release is shown. This clone could be stimulated for IFN-\gamma release by M12.Kb\textsuperscript{wt} or T1.Kb\textsuperscript{wt}, but not by M12.Kb\textsuperscript{m} or T1.Kb\textsuperscript{m} cells.

**CTL precursor frequency data confirm that the mutated form of H-2K\textsuperscript{b} does not engage CD8 to elicit H-2K\textsuperscript{b} alloreactive CTL**

The precursor frequency of CTL reactive with M12.Kb\textsuperscript{wt} and M12.Kb\textsuperscript{m} cells was determined in naive mice and in mice primed in vivo with H-2Kb\textsuperscript{wt} or H-2Kb\textsuperscript{m} incompatible skin grafts (Table II). Primary in vitro stimulation with spleen cells that express H-2Kb\textsuperscript{wt} elicited coreceptor-dependent, H-2K\textsuperscript{b}-specific alloreactive T cells that killed M12.Kb\textsuperscript{wt}, but not M12.Kb\textsuperscript{m}, cells. In contrast, primary in vitro stimulation with the H-2Kb\textsuperscript{m} molecule produced very few H-2K\textsuperscript{b} alloreactive T cells that killed either M12.Kb\textsuperscript{wt} or M12.Kb\textsuperscript{m} cells, suggesting that the mutant H-2Kb\textsuperscript{m} molecule is a very poor primary allo-stimulus. Following in vivo priming with a skin graft expressing H-2Kb\textsuperscript{m}, the frequency of H-2K\textsuperscript{b} alloreactive T cells was increased to a level comparable to that obtained when the skin grafts expressed the wild-type form of H-2K\textsuperscript{b} (Table II). Thus, the mutated form of H-2K\textsuperscript{b}, which does
The precursor frequency of CTL that can kill M12.Kb.m cells is not engaged CD8, is a potent in vivo priming stimulus for eliciting H-2K\textsuperscript{b} specific alloreactive CTL. The CTL generated following in vivo priming with either the wild-type or mutant H-2K\textsuperscript{b} molecule includes CTL that can kill M12.Kb.m cells, and thus do not require TCR/CD8 coengagement. The stimulation with H-2K\textsuperscript{b}-specific alloreactive T cell lines isolated from mice that had been primed with an H-2K\textsuperscript{b}-incompatible skin graft and restimulated in vitro with spleen cells that expressed H-2K\textsuperscript{b}-wt. The CTL lines selected were CD8 dependent, as defined by their ability to kill T2.K\textsuperscript{b}-wt, but not T2.Kb.m cells. Three of these 11 CTL lines could kill M12.Kb.m, but not M12.Kb.m target cells; five lines killed M12.Kb.m cells less efficiently than M12.Kb.wt cells; and three lines (shown in Fig. 6) killed both M12.Kb.wt and M12.Kb.m cells equally well. These data confirm that CD8 bystander-dependent T cells are a component of the secondary alloresponse to a MHC class I disparity.

Discussion

Within the population of CD8\textsuperscript{b} class I alloreactive CTL there are two defined phenotypes. One group of CTL cannot kill in the presence of Ab to CD8 and is thus referred to as CD8 dependent. A second discernible group of CTL is not inhibited by CD8 Ab and is referred to as CD8 independent. These two populations were initially described by MacDonald et al. (29), who demonstrated that while Ab to CD8 inhibited virtually all CTL clones generated in a primary in vitro MLR, killing by some clones isolated following secondary in vitro stimulation was unaffected by Ab to CD8. The ability of the CD8-independent CTL clones to function in the absence of CD8 was attributed to the expression of TCRs with a high affinity for their target alloantigen. Subsequent studies (3, 6) revealed a strict correlation between CD8 independence, as defined by Ab inhibition studies, and the ability to kill cells expressing alloantigenic MHC molecules with a mutation in the CD8 binding region (222–229) of the α3 domain. With respect to CD8 dependence, the alloreactive CTL described herein represent an alternate category of CTL. They were inhibited by Ab to CD8, yet in the absence of Ab could kill cells expressing the α3 domain mutant MHC molecules provided that the target cells also expressed MHC molecules with an intact CD8 binding site. These CTL are thus a subset of the previously defined CD8-dependent population; however, we refer to these cells as CD8 bystander dependent to denote that the binding of CD8 can be with a different MHC class I molecule than that which is bound by the TCR.

We had previously noted that the generation of CTL during a primary in vitro MLR response to stimulator cells expressing the α3 domain mutant class I molecules was either weak or nonexistent. It was therefore surprising that skin grafts in which the only disparity was the mutant class I molecule were rapidly rejected. Furthermore, in vitro restimulation of spleen cells from mice that had rejected such a graft was very effective in generating CTL that were characterized as CD8 bystander dependent. The precursor frequency data also confirmed the development of a strong alloresponse following in vivo priming and in vitro stimulation with the H-2K\textsuperscript{b}m molecule. These CTL could also be isolated following priming with a skin graft that expressed H-2K\textsuperscript{b}m, although the pCTL frequency was lower than that obtained following H-2K\textsuperscript{b}m stimulation in vivo and in vitro. Previous attempts to raise CD8-independent CTL have also involved in vivo priming (29) or stimulation in vitro in the presence of cross-linking Abs to CD8 (30). Alloreactive CTL that have been previously isolated in the absence of such approaches are dependent on CD8 coreceptor activity. The
critical question is why do most class I-restricted T cells require CD8 coreceptor activity, whereas for other T cells CD8 can function as an accessory molecule? In addition, there are T cells that are totally CD8 independent. While many previous studies have assumed that CD8-independent T cells can recognize APCs because their TCR has a high affinity for the target MHC molecule, several studies have shown that, at least for some CD8-dependent T cells, the requirement for CD8 can be overcome by increasing the density of antigenic MHC-peptide complexes on the cell surface (12, 31–35). Thus, perhaps some CD8-independent T cells recognize a ligand present at high density on the target cell. Alloantigenic determinants that are not dependent on a unique peptide or do not require a peptide at all would be examples of determinants that could be expressed at higher levels than conventional peptide-unique epitopes. We have previously described a number of CD8-independent CTL that are capable of recognizing such determinants and, unlike most conventional CD8 coreceptor-dependent H-2K\(^b\) alloreactive CTL, are able to kill T2.K\(^b\) cells. Interestingly, most (10 of 12) of the CD8 bystander-dependent CTL were also able to kill T2.K\(^b\). Whether the ability to recognize high density ligands increases the likelihood that a TCR binding to the mutant molecule is in the vicinity of a CD8 molecule that is bound to a nonantigenic class I molecule is an unknown, yet intriguing, possibility. In contrast, a low occupancy of the TCR would mandate that CD8 be bound by the same MHC molecule and thus function as a coreceptor. We are currently further analyzing the nature of the allo-determinants recognized by CD8-independent and CD8 bystander-dependent alloreactive CTL.

A puzzling issue arising from our observations is why the CD8 bystander cell requires in vivo priming, whereas the CD8 dependent T cell does not. Several studies have documented situations in which activation of naive T cells requires greater stimulation than activation of primed effector cells (36). One possibility is that specialized APC, such as Langerhans cells, at the site of the skin graft are ideal for activation of naive cells, particularly in the setting of an inflammatory response. Perhaps such conditions are more potent than primary in vitro stimulation with spleen cells for the activation of naive T cells and thus are able to activate T cells that express TCR that are able to recognize peptide-independent epitopes with low affinity.

The coreceptor role of CD8 is defined as the requirement for CD8 to engage the same MHC class I molecule as the TCR and has been demonstrated in many systems (3, 6–8, 37). The coreceptor role of CD8 provides two functions in T cell activation: 1) the binding of CD8 to class I activates the kinase p56\(^{lck}\) (38, 39), which, in association with TCR-mediated signal transduction events, augments signaling pathways; and 2) CD8 adds to the affinity of the TCR/MHC interaction. In this model, the CD8-mediated transduction events only occur when CD8 engages an MHC class I molecule in conjunction with the TCR. CD8-mediated signal transduction events are required for serine esterase release, hydrolysis of phosphatidylinositides, and an increase in intracellular Ca\(^{2+}\) (9–11) in a system in which CD8 was bound to immobilized, nonantigenic class I molecules subsequent to Ab-induced TCR ligation. Furthermore, we have found that coengagement of CD8 and the TCR is essential for the hydrolysis of phosphatidylinositides (PI\(^3\)) in CD8-dependent CTL (12). Moreover, in CD8-independent CTL, although CD8 engagement is not required for cytolyis, it is required for PI hydrolysis. Interestingly, in these

Table II. Frequency of anti-H-2K\(^b\) alloreactive spleen cells in CD2F\(_1\) mice following a 7-day MLR with stimulator spleen cells expressing either wild-type H-2K\(^b\) or the mutant H-2K\(^b\) that does not engage CD8

<table>
<thead>
<tr>
<th>Graft(^a)</th>
<th>Stimulator(^b)</th>
<th>pCTL (1/...) for M12.K(^b).wt</th>
<th>pCTL (1/...) for M12.K(^b).m</th>
<th>pCTL (1/...) for T1.K(^b).wt</th>
<th>pCTL (1/...) for T1.K(^b).m</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>28.1 (K(^b).wt)</td>
<td>Not detectable</td>
<td>17,634 ± 7,436</td>
<td>210,723 ± 74,515</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>29.7 (K(^b).m)</td>
<td>Not detectable</td>
<td>12,179 ± 6,663</td>
<td>210,723 ± 74,515</td>
<td></td>
</tr>
<tr>
<td>28.1 (K(^b).wt)</td>
<td>28.1 (K(^b).m)</td>
<td>216,423 ± 41,313</td>
<td>15,690 ± 4,638</td>
<td>6,047 ± 3,828</td>
<td></td>
</tr>
<tr>
<td>29.7 (K(^b).m)</td>
<td>29.7 (K(^b).m)</td>
<td>15,684 ± 11,334</td>
<td>6,047 ± 3,828</td>
<td>4,380</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) CD2F\(_1\) mice were grafted with 28.1 (transgenic for wild-type H-2K\(^b\), denoted K\(^b\).wt) or 29.7 (transgenic for mutant H-2K\(^b\) that does not engage CD8, denoted K\(^b\).m) trunk skin. Spleen cells were harvested 14 days after skin grafting. At this time, the grafts were completely rejected.

\(^b\) Stimulators for the culture were either 28.1 (H-2K\(^b\).wt) or 29.7 (H-2K\(^b\).m) spleen cells. Some cultures were a primary anti-H-2K\(^b\) in vitro stimulation of cells from naive mice, and other cultures were a secondary in vitro stimulation of spleen cells from mice that received an H-2K\(^b\) (wild-type or mutant)-incompatible skin graft.
CD8-independent CTL, TCR and CD8 coengagement was not required for PI hydrolysis providing that the target cell also expressed a nonantigenic class I molecule to which CD8 could bind (12). Thus, these CD8-independent CTL were CD8 bystander dependent for PI hydrolysis. Furthermore, even though the TCR expressed by these CD8-independent CTL provided sufficient affinity/avidity for killing, the signaling pathway leading to phosphatidylinositol bisphosphate hydrolysis was absolutely dependent on CD8 engagement. Therefore, T cell functions dependent upon this signaling pathway require CD8 activation regardless of the affinity of the TCR for its ligand.

Whether the primary role of CD8 is to increase the affinity of the TCR/MHC interaction is controversial. A recent study directly demonstrated that CD8 effectively increases the duration and extent of TCR engagement by stabilizing the TCR/MHC complex once it is formed (40). Other experiments using CD8 blocking Abs indicate that the relationship between the individual TCR and CD8 molecules is more complex than CD8 simply adding affinity to this interaction (41, 42). For example, it has been demonstrated that binding of the 2C CTL clone to H-2Ld in association with the p56lck that either act independently of or intersect downstream with TCR-mediated signal transduction. Currently, there is substantial evidence to indicate that following initial TCR/CD8 coengagement, binding of CD8 to other MHC class I molecules increases the overall avidity of the T cell-target cell interaction. This evidence includes studies that demonstrate that cross-linking the TCR with soluble mAb induces the binding of alloreactive CTL to immobilized class I molecules of any MHC allelic form (9–11, 48). This binding of T cells to the immobilized MHC class I is inhibited by Ab to CD8. According to a model proposed to explain these observations, an initial activation stimulus through the TCR converts CD8 to a high affinity state (9). In these studies, Ab to the TCR or CD3 was used as the activating stimulus. In a physiologic setting, however, activation of most CTL requires the coengagement of TCR and CD8 by the same MHC class I molecule (6). Recently, the two events, 1) coengagement of CD8 and the TCR, and 2) binding of CD8 to other MHC class I molecules, have been clearly separated. In this scheme (49) the Glu227—lysine mutation in the MHC class I α3 domain inhibits the initial binding by CD8 and, hence, TCR and CD8 coengagement. This mutation has no effect on the CD8/MHC interaction subsequent to the activation through the TCR and CD8 coengagement event that converts CD8 to the high affinity state. It is unclear whether the high affinity CD8 overcomes the effect of the mutation by binding to the 222–229 loop or at the other regions of the class I molecule that contact CD8 (50–53). Thus, in T cells activated with Abs to the TCR, CD8 can bind to nonantigenic class I molecules even if they contain the mutation at residue 227 (49). Therefore, CD8 can mediate an overall increase in T cell/target cell avidity through a generalized class I binding following the activation of CD8 when CD8 is coengaged with the TCR. This coengagement of the TCR and CD8, however, is dependent on the binding site in the α3 domain.

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References


