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Activation Requirements, Lytic Mechanism, and Development of a Novel Anti-CD8-Resistant CTL Population[1]

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Almost all conventional CD8$^+$ CTL and their CD8$^+$ precursors are inhibited by anti-CD8 mAb. This requirement for CD8 function reflects both an avidity-augmentation role and a signal-transduction role for CD8 on T cells. We have, however, previously identified and partially characterized a novel functional population of CD8$^+$, but anti-CD8-resistant, MHC class I-allospecific CTL. These CTL have unusual activation requirements in that their efficient generation in vitro requires inhibition of the CD8 avidity contribution (but not the CD8 signaling contribution), by anti-CD8 mAb. In this study, we have investigated the relationship of anti-CD8-sensitive and anti-CD8-resistant CTL by several criteria. These CTL populations share the phenotypic markers we have tested to date, they have similar but not identical Ag-specific repertoires, and they both appear to be generated from naive unprimed T cells. However, anti-CD8-sensitive and anti-CD8-resistant CTL populations exhibit important functional differences. They differ in their kinetics of activation in vitro, their dependence on exogenous cytokines, their use of lytic effector mechanisms, and their tissue distribution during ontogeny. Based on these results, we favor the hypothesis that these CTL populations represent distinct T cell lineages or subsets, and not merely different TCR avidity ranges within a single T cell lineage or subset. ***The Journal of Immunology,*** **1998, 160: 2715–2724.**

Activation of a functional CTL response plays a central role in the rejection of allogeneic organ/tissue transplants: many reports indicate that grafts able to activate CTL are rejected, whereas grafts unable to activate CTL are not rejected (1–7). CTL can also play pivotal roles in graft-vs-host disease (8, 9), autoimmunity (10, 11), antiviral responses (12), and antitumor responses (13).

Almost all conventional CD8$^+$ CTL and their CD8$^+$ precursors (pCTL)[3] are inhibited by anti-CD8 mAb (14–16). This requirement for CD8 function reflects both an avidity-augmentation role and a signal-transduction role for CD8 on T cells. We have, however, previously identified and partially characterized a subset of CD8$^+$ MHC class I-allospecific precursor and effector CTL that are activated in the presence of anti-CD8 mAb in vitro (17, 18). These CTL may require anti-CD8 mAb to reduce their avidity for alloantigen to an appropriate TCR-triggering range for functional activation in in vitro cultures, in which Ag is extremely abundant (17). Berzofsky and colleagues have demonstrated recently that very low doses of Ag can be used to selectively activate high avidity CTL in vitro (19, 20). In that model, low Ag availability would limit the number of TCR engaged, and may mimic a low/moderate avidity stimulus. The use of either anti-CD8 mAb or very low Ag doses is thought to spare the high avidity cells from overstimulation that would otherwise lead to inactivation and/or cell death (17, 20).

In vitro, anti-CD8-resistant CTL effector cells are actively induced from high frequency pCTL by alloantigen only in the presence of an anti-CD8 mAb that can multivalently cross-link CD8 on the pCTL surface (17, 18). Simple blockade of CD8 (with bivalent anti-CD8 mAb in the absence of cross-linking) or elimination of competing anti-CD8-sensitive pCTL (in limiting dilution analysis assays) is not sufficient to induce or permit the generation of these novel CTL effector cells (17). Thus, although anti-CD8-resistant pCTL appear not to require CD8 avidity contributions, they do appear to require CD8 signaling contributions initiated by multivalent cross-linking of CD8.

A similar population of CD8$^+$ CTL can be activated by an MHC class I-disparate allogeneic skin graft after in vivo anti-CD8 mAb-mediated depletion of the vast majority of CD8$^+$ T cells (6). These novel in vivo effectors cause the rapid rejection of the allogeneic graft, and exhibit allospecific anti-CD8-resistant CTL activity in vitro after graft rejection (6). High avidity effectors such as these CTL may be critical for many in vivo immune responses, in which Ag may be limiting (19, 21–23).

The anti-CD8-resistant status of these novel CD8$^+$ CTL effector cells raises the issue of their lineage relationship to conventional anti-CD8-sensitive CD8$^+$ CTL and to other effector cells with lytic activity. One hypothesis is that anti-CD8-resistant CTL simply represent the rare, very highest avidity clones within the conventional CD8$^+$ CTL lineage (19, 20). Anti-CD8-resistant MHC class I-allospecific CTL are CD8$^+$, CD4$^-$, Thy-1$^+$, Ly-6$^+$, LFA-1$^+$, as are conventional anti-CD8-sensitive MHC class I-allospecific CTL (17, 18). This phenotyping information is consistent with, but does not prove, a single lineage for these two CTL types. In contrast, our limiting dilution analyses demonstrated that the CTL precursor frequencies for anti-CD8-sensitive and anti-CD8-resistant CTL are comparable, demonstrating that anti-CD8-resistant CTL are not a rare subset of CTL (17).

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[3] Abbreviations used in this paper: pCTL, CTL precursor; Con A SN, Con A-induced supernatant; FasL, Fas ligand; IEL, intraepithelial lymphocytes; mHag, minor histocompatibility Ag.

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An alternative hypothesis is that anti-CD8-resistant CTL represent a distinct effector T cell lineage, analogous to lytic effector lineages in the IEL population, the liver, and other secondary lymphoid organs (24–29). This "separate lineage" hypothesis does not exclude the possibility that anti-CD8-resistant CTL do indeed have high avidity for Ag, since they apparently do not require the CD8 avidity contribution. Thus, direct TCR/MHC-peptide avidity measurements, even if they were possible for intact responder-stimulator pairs, would not distinguish between the one lineage and two lineages hypotheses.

To test these alternative hypotheses, we therefore undertook a more extensive characterization of the phenotype, repertoire, in vitro activation requirements, lytic effector mechanisms, and tissue distributions during ontogeny of the novel anti-CD8-resistant CTL. Based on the results from these studies, we conclude that the anti-CD8-resistant pCTL/CTL we have identified represent a distinct T cell lineage or subset.

Materials and Methods

Mice

Mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or Charles River Laboratories (Wilmington, MA), or were bred in our animal facility. BALB/c (H-2^d), C57BL/6 (B6; H-2^b, *fas*$^+$, perforin$^+$, Thy-1.2), B6.PL-Thy-1^a/Cy (H-2^b, Thy-1.1), DBA/2 (H-2^d), B6.C-H-2^{bm1}/ByJ (bm1; H-$2K^{bm1}$ mutant), C3H.SW (H-2^b), B6.MRL-Fas^{lpr} (B6.lpr; H-2^b, Fas$^-$, perforin$^+$), and C57BL/6-Pfp^{tm1} (B6.Perf-KO; H-2^b, Fas$^+$, perforin$^-$) mice were used.

Con A-induced supernatant (Con A SN)

Con A SN was the 18-h supernatant from Con A-stimulated BALB/c or B6 spleen cells prepared as described (30). The Con A SN was supplemented with 0.2 M α-methyl-D-mannoside to neutralize residual Con A and was used in MLC at a final concentration of 12.5 to 25%.

Abs and cell lines

83-12-5 anti-CD8α (mouse IgM mAb), 53-6.72 anti-CD8α (rat IgG2a mAb), 3.155 anti-CD8α (rat IgM mAb), 53-5.83 anti-CD8β (rat IgG1 mAb), 145-2C11 anti-CD3 (hamster IgG mAb), H57-597 anti-TCR-$\alpha\beta$ (hamster IgG mAb), HO-22-1 anti-Thy-1.1 (rat IgM mAb), and 30-H12 anti-Thy-1.2 (rat IgG2b mAb) were used as hybridoma cell line culture supernatants or mAb purified from ascites fluid. P815 (H-2^d) and EL4 (H-2^b) cell lines were maintained in vitro for use as target cells, where indicated.

In vitro generation of CTL

MLC of 2.5 to 5 $\times$ 10^6 spleen, lymph node, or thymus cells from primed or normal unprimed responder mice and 5 $\times$ 10^6 irradiated (2000 R) stimulator spleen cells were established in 2 ml complete RPMI 1640 medium supplemented with glutamine, nonessential amino acids, sodium pyruvate, antibiotics, 2-ME, and 5% FCS, as previously described (17, 18). Con A SN was included in all response cultures, unless otherwise indicated, to provide exogenous Th cell-derived lymphokines necessary for CTL maturation and/or proliferation. In addition, the induction cultures contained anti-CD8 mAb, where indicated. All cultures were incubated at 37°C in 7.5% CO_2 humidified air. On days 3 to 7, as indicated, cells from the cultures were collected, washed, counted, and assayed for CTL activity by their ability to lyse ^{51}Cr-labeled splenic LPS blast target cells or tumor target cells in a 4-h ^{51}Cr release assay. CTL were assayed in triplicate at each of four E:T ratios. When anti-CD8 mAb or other mAbs were included in the 4-h lytic effector assay, the CTL effector cells were preincubated in the mAb for 10 to 30 min before labeled target cells were added to the effectors; mAb was also present throughout the 4-h assay. Percent specific lysis = 100 $\times$ (experimental release − spontaneous release)/(maximum release − spontaneous release). SDs were routinely less than 5%, and are omitted from the figures. Lysis of responder strain targets was routinely <10%.

In vivo priming

Adult mice were injected i.p. once with 1 to 2 $\times$ 10^7 normal spleen cells from minor histocompatibility Ag (mHag)-disparate donor mice in 0.5 to 1 ml PBS. At least 3 wk later, the primed mice were killed and their spleen cells were used as responder cells in MLC.

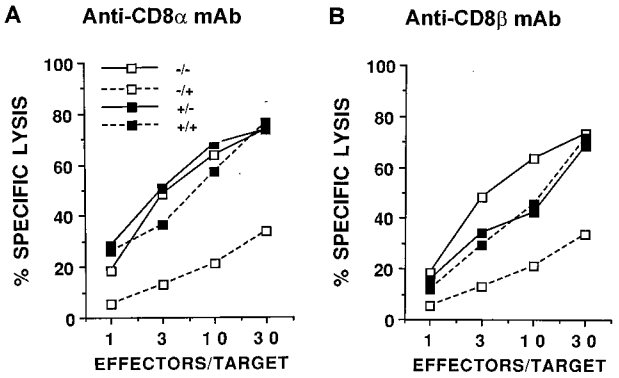

FIGURE 1. Anti-CD8-resistant CD8$^+$ CTL can be induced by either anti-CD8α or anti-CD8β mAb in MLC. B6 splenic responder cells were cocultured for 5 days with DBA/2 splenic stimulator cells, either with (solid symbols) or without (open symbols) anti-CD8α mAb (*A*) or anti-CD8β mAb (*B*). On day 5, effector cells were tested for lytic activity against P815 targets in the presence (dotted lines) or absence (solid lines) of anti-CD8α mAb. The following code is used in all figures: −/− = no mAb in MLC, no mAb in target lysis assay; −/+ = no mAb in MLC, anti-CD8 in target lysis assay; +/− = anti-CD8 in MLC, no mAb in target lysis assay; and +/+ = anti-CD8 in MLC, anti-CD8 in target lysis assay.

Effector cell depletions

Single cell suspensions of effector cells generated in MLC were treated at 1 $\times$ 10^7 cells/ml with anti-Thy-1.1 mAb or anti-Thy-1.2 mAb for 30 min at 4°C, after which the cells were pelleted and resuspended in appropriately diluted guinea pig complement (Life Technologies, Grand Island, NY) and incubated for 40 min at 37°C. After extensive washing, the cells were reconstituted, without recounting, to the desired concentration based on pretreatment cell counts for use in CTL effector assays.

Results

We have demonstrated previously that adult murine splenic T lymphocytes contain a population of CD8$^+$ MHC class I-allospecific precursor cells that can be induced to generate CD8$^+$ anti-CD8-resistant CTL effector cells in MLC stimulation cultures (17, 18). Figure 1*A* illustrates this finding. Unprimed adult B6 responder spleen cells were cocultured with MHC-disparate DBA/2 stimulator cells in MLC supplemented with Con A SN to provide a source of Th cell-derived cytokines, in the presence or absence of anti-CD8α mAb. CTL effector function was assessed on P815 target cells, which express MHC class I, but not class II alloantigens. The CTL lytic activity generated in MLC in the absence of anti-CD8α mAb was sensitive to inhibition by anti-CD8α mAb during the target lysis assay. In contrast, the CTL lytic activity generated in MLC in the presence of anti-CD8α mAb was largely resistant to inhibition by anti-CD8α mAb during the target lysis assay. In some, but not all experiments, we noted that CTL activity for the +/− group was somewhat stronger than the CTL activity for the +/+ group. This may reflect some leak-through of conventional anti-CD8-sensitive CTL during their induction in the MLC in the presence of anti-CD8 mAb; these CTL are then inhibited by additional fresh anti-CD8 mAb during the lytic assay. However, the critical comparison to be made between groups is between the −/+ group (i.e., the lack of appreciable anti-CD8-resistant CTL after induction in the absence of anti-CD8 mAb) and the +/+ group (i.e., the presence of appreciable anti-CD8-resistant CTL after induction in the presence of anti-CD8 mAb).

We began our characterization of the anti-CD8-resistant CTL by determining their CD8 phenotype. Some subsets of CD8$^+$ T cells

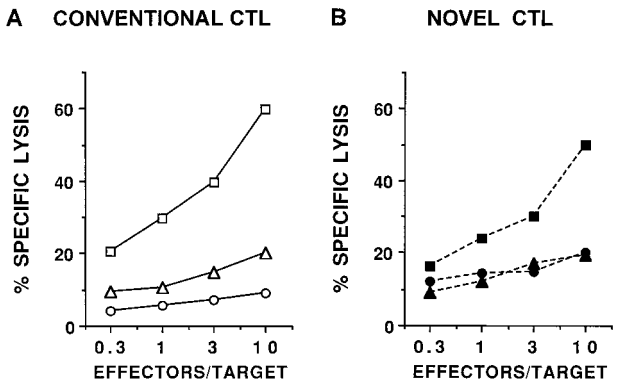

FIGURE 2. Anti-CD8-resistant CTL express the $\alpha\beta$ form of the TCR. bm1 splenic responder cells were cocultured for 5 days with B6 splenic stimulator cells, either with (*B*) or without (*A*) anti-CD8α mAb. On day 5, effector cells were tested for lytic activity against EL4 targets in the presence (*B*) or absence (*A*) of anti-CD8α mAb; thus, *A* represents $-/-$ conditions, and *B* represents $+/+$ conditions. In each panel, target lysis was tested in the absence (squares) or presence of additional mAbs: anti-CD3 mAb (circles) or anti-TCR-$\alpha\beta$ mAb (triangles).

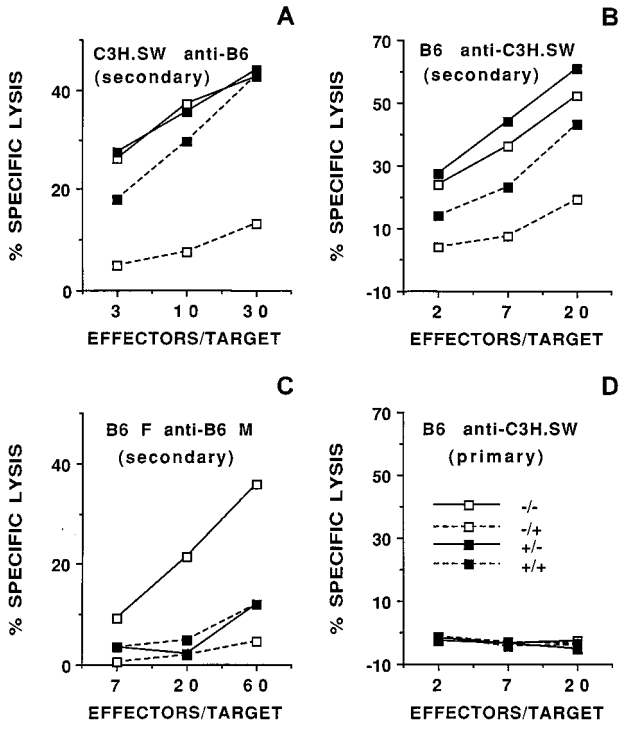

FIGURE 3. Anti-CD8-resistant CTL can be generated against mHags. Spleen cells from in vivo primed (*A–C*) or unprimed mice (*D*) were cocultured for 5 days with mHag-disparate splenic stimulator cells in the presence (solid symbols) or absence (open symbols) of anti-CD8 mAb. On day 5, effector cells were tested for lytic activity against stimulator strain targets in the presence (dotted lines) or absence (solid lines) of anti-CD8 mAb.

express a CD8$\alpha\alpha$ homodimer rather than a CD8$\alpha\beta$ heterodimer, although the functional significance of this difference is not fully understood (31, 32). Since anti-CD8 mAb blocking cannot be used to phenotype the anti-CD8-resistant CTL (we had shown previously that these CTL are resistant to blocking of lytic effector function by either anti-CD8α mAb or anti-CD8β mAb; Ref. 18), we instead compared the effects of anti-CD8α mAb (Fig. 1*A*) and anti-CD8β mAb (Fig. 1*B*) during the MLC induction culture. These mAbs induced comparable MHC class I-allospecific anti-CD8-resistant CTL generation, demonstrating the CD8$\alpha\beta^+$ phenotype of this T cell population; the slightly stronger activity of anti-CD8α mAb in the experiment shown was not consistently observed (data not shown).

We also determined the TCR phenotype of the CD8$^+$ anti-CD8-resistant CTL. We generated H-2K^b-specific CTL in bm1 anti-B6 MLC, and tested them for lytic activity on FcR$^-$ EL4 target cells. As shown in Figure 2*A*, target cell killing by conventional anti-CD8-sensitive CTL is blocked by either anti-CD3 mAb or anti-TCR-$\alpha\beta$ mAb; thus, these CTL are TCR-$\alpha\beta^+$. Figure 2*B* illustrates that target cell killing by anti-CD8-resistant CTL is also blocked by either anti-CD3 mAb or anti-TCR-$\alpha\beta$ mAb; thus, these CTL are also TCR-$\alpha\beta^+$. The results presented in Figures 1 and 2 exclude the possibility that the anti-CD8-resistant splenic pCTL/CTL belong to the CD8$\alpha\alpha^+$ subset or the TCR-$\gamma\delta^+$ lineage normally associated with IEL and/or CD4$^-$8$^-$ T cells, and establish that these CTL express phenotypic markers typical of most splenic CTL.

As a second approach to comparing conventional and novel CTL, we examined their Ag-specific repertoires. In previous studies, we had focused exclusively on the generation of MHC class I-allospecific CTL in primary stimulation cultures. In the current study, we investigated whether anti-CD8-resistant CTL can be generated against mHags, as a model for self MHC-restricted recognition of viral and tumor Ags. In vivo priming followed by in vitro MLC restimulation in the presence of anti-CD8 mAb induced anti-CD8-resistant MHC class I-restricted mHag-specific CTL in both the B6 anti-C3H.SW and the C3H.SW anti-B6 strain combinations (Fig. 3, *A* and *B*). These "multiple minor"-disparate strain combinations present several or many mHags to the responding T cells; any or all of these mHags might be recognized by anti-CD8-resistant CTL. In contrast to our results with these strain combinations, we were repeatedly unable to generate HY-specific anti-CD8-resistant CTL by B6 female anti-B6 male priming and MLC restimulation in the presence of anti-CD8 mAb; whereas MLC restimulation in the absence of anti-CD8 mAb induced conventional anti-CD8-sensitive CTL, restimulation in the presence of anti-CD8 mAb blocked CTL generation (Fig. 3*C*). The most likely explanation for this is that the anti-CD8 mAb blocked the activation of HY-specific anti-CD8-sensitive CTL, and there were no primed HY-specific anti-CD8-resistant CTL available for activation. In an HY-specific stimulation, a very limited set of mHags is presented to the responding T cells (33); apparently none of these mAgs could be recognized by the anti-CD8-resistant pCTL/CTL population. Thus, anti-CD8-sensitive CTL and anti-CD8-resistant CTL exhibit overlapping, but not identical, Ag-recognition repertoires.

We examined the activation requirements of anti-CD8-resistant mHag-specific CTL. Figure 3, *A* and *B*, illustrates that in vitro restimulation of in vivo primed T cells in the absence of anti-CD8 mAb did not generate anti-CD8-resistant CTL effector function. This result demonstrates that conventional memory CTL responses to these mHags are not anti-CD8 resistant. Furthermore, generation of both anti-CD8-sensitive and anti-CD8-resistant mHag-specific CTL responses required in vivo priming (Fig. 3, *B* and *D*). Together, these results indicate that the mHag-specific anti-CD8-resistant CTL we have identified are not merely inadvertently primed memory conventional CTL.

We also investigated whether the MHC-allospecific anti-CD8-resistant CTL generated in primary in vitro MLC have other properties expected of memory T cells. To do so, we compared the kinetics of activation and the cytokine requirements of CTL generation in MLC in the presence and absence of anti-CD8 mAb. It

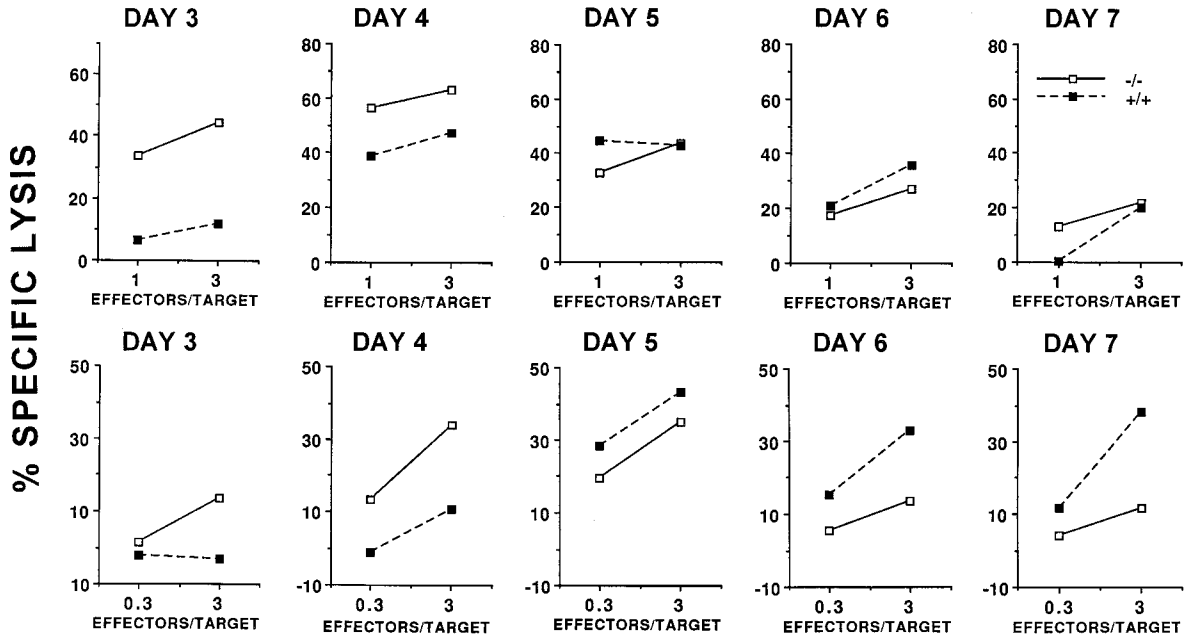

FIGURE 4. Anti-CD8-sensitive CTL and anti-CD8-resistant CTL differ in their activation kinetics in MLC. B6 anti-bm1 (*top panels*) or bm1 anti-B6 (*bottom panels*) MLC of splenic responder and stimulator cells were cocultured for 3 to 7 days, either with (solid symbols) or without (open symbols) anti-CD8 mAb. On days 3 to 7 of each experiment, effector cells were tested for lytic activity against stimulator strain targets in the presence (dotted lines) or absence (solid lines) of anti-CD8 mAb; only the −/− and +/+ effector groups are shown.

is generally thought that memory T cell responses are accelerated and require less help, compared with primary responses. Figure 4 illustrates the results from two kinetics assays in which MHC class I-disparate MLC stimulation cultures were tested for CTL effector function on days 3 to 7. Conventional anti-CD8-sensitive CTL from MLC without mAb were first readily detected on day 3 or 4 of culture. In contrast, anti-CD8-resistant CTL from MLC with anti-CD8 mAb were undetectable at the early time points, and peaked 1 to 2 days after conventional anti-CD8-sensitive CTL generated in the absence of anti-CD8 mAb. Thus, anti-CD8-resistant CTL exhibit somewhat delayed, rather than accelerated, stimulation kinetics compared with anti-CD8-sensitive CTL. This is not due to a lower precursor frequency for anti-CD8-resistant CTL, since we had demonstrated previously by limiting dilution analysis that the two CTL types have comparable precursor frequencies (17).

As an assay for cytokine requirements by the two CTL types, we established MHC class I-disparate MLC stimulation cultures with or without Con A SN as a source of exogenous Th cell-derived cytokines. A typical experiment is shown in Figure 5. Omission of Con A SN, so that CTL generation is dependent on cytokines produced within the culture, usually had a moderate weakening effect on conventional CTL generation in MLC without anti-CD8 mAb (compare −/− groups in *panels A* and *B*). In contrast, omission of Con A SN usually led to a complete failure to generate anti-CD8-resistant CTL in MLC with anti-CD8 mAb (compare +/+ groups in *panels A* and *B*). Among 10 experiments in which CTL generation was induced in the absence of Con A SN, the pattern illustrated in Figure 5 (anti-CD8-sensitive CTL, but no anti-CD8-resistant CTL generated in the absence of Con A SN) was observed in seven cases; in the remaining three experiments, both CTL populations were generated. We never observed anti-CD8-resistant CTL without anti-CD8-sensitive CTL in the absence of Con A SN. Thus, anti-CD8-resistant CTL generation appears to require more or different cytokines than does anti-CD8-sensitive CTL generation, and more or different cytokines than are normally produced and secreted in an MHC class I-disparate MLC with our culture conditions. Addition of an allogeneic MHC class II stimulus to the allogeneic MHC class I stimulus, either on the same stimulator cell (two-cell experiment) or on a separate stimulator cell (three-cell experiment) did not circumvent the requirement for exogenous cytokines for anti-CD8-resistant CTL generation in vitro (data not shown). It should be noted that the successful in vivo priming of these CTL (Fig. 3) indicates that sufficient cytokines are available under physiologic conditions in vivo. Thus, both the activation requirements (Figs. 3 and 5) and the response kinetics (Fig. 4) of anti-CD8-resistant CTL suggest that these effector cells are neither memory/primed conventional CTL nor high avidity naive conventional CTL.

As an additional test of the hypothesis that anti-CD8-sensitive and anti-CD8-resistant CTL instead represent distinct effector lineages or subsets, we investigated the lytic mechanisms used by

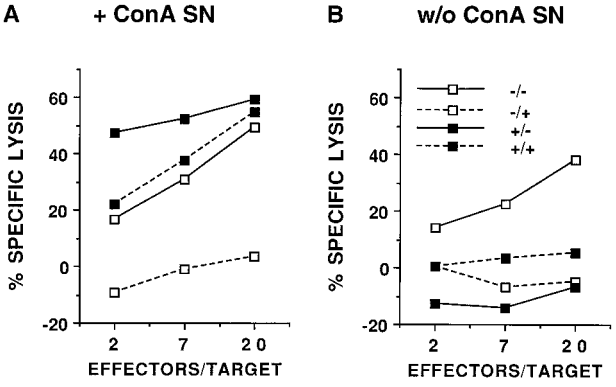

FIGURE 5. Anti-CD8-sensitive CTL and anti-CD8-resistant CTL differ in their cytokine requirements in MLC. B6 splenic responder cells were cocultured with bm1 splenic stimulator cells for 5 days, either with (solid symbols) or without (open symbols) anti-CD8 mAb. On day 5, effector cells were tested for lytic activity against bm1 targets in the presence (dotted lines) or absence (solid lines) of anti-CD8 mAb.

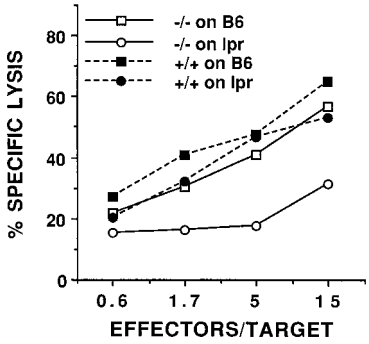

FIGURE 6. Anti-CD8-sensitive CTL and anti-CD8-resistant CTL differ in their use of Fas/FasL interactions for target cell lysis. bm1 splenic responder cells were cocultured for 5 days with B6 splenic stimulator cells, either with (solid symbols) or without (open symbols) anti-CD8 mAb. On day 5, effector cells were tested for lytic activity in the presence (dotted lines) or absence (solid lines) of anti-CD8 mAb; only the −/− and +/+ effector groups are shown. Targets were either B6 (Fas^+, squares) or B6.lpr (Fas^-, circles).

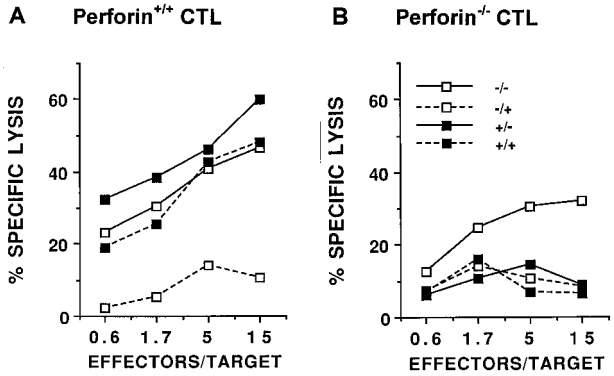

FIGURE 7. Anti-CD8-sensitive CTL and anti-CD8-resistant CTL differ in their requirement for the perforin lytic mechanism. B6 ($perforin^{+/+}$; *A*) or B6.Perf-KO ($perforin^{-/-}$; *B*) splenic responder cells were cocultured for 5 days with bm1 splenic stimulator cells, either with (solid symbols) or without (open symbols) anti-CD8 mAb. On day 5, effector cells were tested for lytic activity against bm1 targets in the presence (dotted lines) or absence (solid lines) of anti-CD8 mAb.

these T cells. Conventional CTL have been reported to utilize both a Fas/FasL-dependent lytic mechanism and a perforin-dependent lytic mechanism. These two mechanisms appear to be independent but additive in their effects (34–36), and may require somewhat different TCR-initiated intracellular signaling events (37). Other effector populations have been reported to rely more heavily on one or the other of these lytic mechanisms (38–40). To examine the use of the Fas/FasL system, we tested anti-CD8-sensitive and anti-CD8-resistant CTL for their abilities to lyse Fas^+ and Fas^- target cells. H-$2K^b$-allospecific CTL generated in MLC in the presence or absence of anti-CD8 mAb were tested for lysis of B6 (H-$2K^b$, Fas^+) and B6.lpr (H-$2K^b$, Fas^-) target cells. Conventional anti-CD8-sensitive CTL exhibited lysis of Fas^+ targets, and significantly reduced but still detectable lysis of Fas^- targets (Fig. 6, −/− effector groups). This result confirms that conventional CTL utilize the Fas/FasL lytic mechanism, but also utilize a Fas/FasL-independent lytic mechanism. In contrast to conventional CTL, anti-CD8-resistant CTL from anti-CD8 mAb-containing MLC usually lysed Fas^+ and Fas^- targets nearly equally well (Fig. 6, +/+ effector groups), indicating that these novel $CD8^+$CTL do not normally utilize the Fas/FasL mechanism for target cell lysis.

The lack of dependence of anti-CD8-resistant CTL on the Fas/FasL lytic mechanism led to the prediction that these effector T cells would be profoundly dependent on the perforin lytic mechanism. We tested this prediction using $perforin^+$ and $perforin^-$ responding T cell populations for CTL generation in MLC. B6 ($perforin^+$) and B6.Perf-KO ($perforin^-$) spleen cells were cocultured in MLC with bm1 stimulator cells in the presence or absence of anti-CD8 mAb (Fig. 7). As expected, lack of perforin in the responding T cell population had a moderate weakening effect on conventional anti-CD8-sensitive CTL generation in MLC without anti-CD8 mAb (compare −/− effector groups in *panels A* and *B*). The residual perforin-independent lytic activity of these CTL confirms that the Fas/FasL mechanism is operative in these conventional CTL, in agreement with the conclusions from Figure 6. In contrast to the results for anti-CD8-sensitive CTL, lack of perforin in the responding T cell population completely prevented the generation of anti-CD8-resistant CTL activity in MLC with anti-CD8 mAb (compare +/+ effector groups in *panels A* and *B*). The lack of anti-CD8-resistant CTL activity in B6.Perf-KO cells indicates an almost total dependence of this effector T cell population on the perforin lytic mechanism, and is in agreement with the conclusions from Figure 6. Together, these two complementary experimental strategies demonstrate that conventional anti-CD8-sensitive CTL and the novel anti-CD8-resistant CTL we have identified differ in their use of lytic effector mechanisms.

These two CTL populations also differ in their tissue distributions in normal adult mice. Both splenic (Fig. 8*A*) and mesenteric lymph node (Fig. 8*B*) responder cells contain the anti-CD8-resistant pCTL/CTL population. In contrast to the peripheral lymphoid tissues, adult thymocytes did not generate anti-CD8-resistant CTL (Fig. 8*C*). Adult B6 thymic responder cells and bm1 stimulator cells were cocultured under conditions identical to those used in the splenic and lymph node responder stimulation cultures, in the same experiment. Thymic responder cells generated bm1-specific CTL activity in MLC in the absence of anti-CD8 mAb; this CTL activity was sensitive to inhibition by anti-CD8 mAb in the target lysis assay. However, thymic responder cells did not generate bm1-specific CTL activity in the presence of anti-CD8 mAb. This result suggests that generation of conventional anti-CD8-sensitive CTL response was inhibited by anti-CD8 mAb in the MLC, and that an alternative anti-CD8-resistant CTL response was not induced from thymic responders.

This difference between adult peripheral and thymic pCTL/CTL activities was not unique to the bm1-specific response. B6 responder cells were cocultured in MLC with BALB/c stimulator cells, or BALB/c responder cells were cocultured in MLC with B6 stimulator cells. In each case, the splenic responders, but not thymic responders, generated anti-CD8-resistant CTL effector cells when anti-CD8 mAb was present in the MLC (data not shown; we have generated anti-CD8-resistant splenic CTL against many individual MHC class I disparities; Refs. 17 and 18). We have used several anti-CD8 mAbs, including an IgG anti-CD8β mAb and two different IgM anti-CD8α mAbs in MLC. Each of these anti-CD8 mAbs induced anti-CD8-resistant CTL from splenic, but not from thymic, responder cell populations. These results indicate that adult peripheral pCTL contain a functional population, the anti-CD8-resistant pCTL subset, that cannot be demonstrated in adult thymic pCTL.

We reasoned that either the thymus truly lacks this pCTL population, or this population is present but is not activated in the MLC. It was possible that a regulatory/suppressive cell in the adult thymic responder population inhibits the activation of otherwise competent anti-CD8-resistant pCTL. Figure 9 presents results from

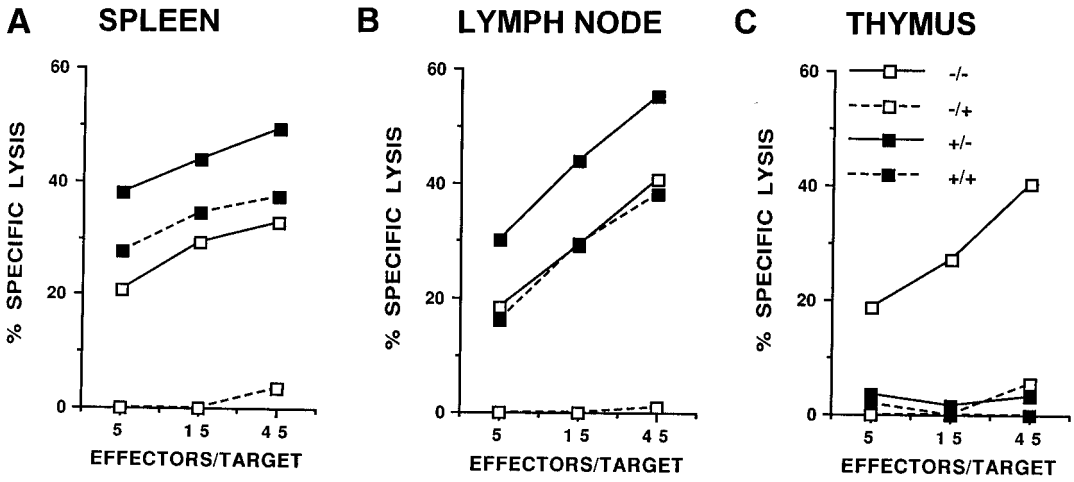

FIGURE 8. In the presence of anti-CD8 mAb, anti-CD8-resistant CTL develop in MLC from peripheral, but not from thymic adult T cells. B6 responder cells (2.5×10^6/well) from spleen, lymph node, or thymus (*C*) were cocultured for 5 days with bm1 splenic stimulator cells (5×10^6/well), either with (solid symbols) or without (open symbols) 83-12-5 anti-CD8α mAb. On day 5, effector cells were tested for lytic activity against bm1 targets in the presence (dotted lines) or absence (solid lines) of anti-CD8 mAb.

an experiment investigating this possibility. Splenic and thymic adult B6 responder cells were mixed, and then cocultured in MLC with BALB/c stimulator cells in the presence of anti-CD8 mAb (Fig. 9*C*). Thymocytes did not interfere with the generation of splenic anti-CD8-resistant CTL in these mixed responder cultures. This result makes a regulatory or suppressive explanation unlikely, although it does not exclude the more restrictive possibility that a suppressive effect acts only on thymic, and not on splenic, pCTL.

Alternatively, it was possible that a non-CTL lineage cell, such as a helper cell, was limiting in the adult thymic responder cultures. We added Con A SN to all stimulation cultures, in concentrations sufficient to provide the Th cell-derived lymphokines required for the generation of thymic conventional, anti-CD8-sensitive CTL. Nevertheless, if anti-CD8-resistant pCTL need some additional signal or lymphokine, the thymic defect could be in the helper cell rather then the CTL lineage. Figure 10 shows the results of an experiment in which we investigated whether adult splenic responder cells could provide this postulated helper effect and reveal anti-CD8-resistant CTL generation from adult thymic pCTL. B6-Thy-1.2 spleen cells and B6.Pl-Thy-1.1 congenic thymus cells (and the reciprocal pairs) were mixed, and then cocultured with bm1 stimulator cells in the presence of anti-CD8 mAb. After the 5-day MLC, the effector cells generated were treated with anti-Thy-1.1 or anti-Thy-1.2 mAb plus complement, to phenotype the anti-CD8-resistant CTL effector populations and identify their splenic or thymic origin. In each of the mixed responder populations, virtually all of the anti-CD8-resistant CTL activity was derived from the splenic precursor population. Thus, adult splenic responder cells did not rescue an adult thymus-derived anti-CD8-resistant pCTL population in these cultures.

The results shown above indicate that the adult thymocyte population is deficient in anti-CD8-resistant pCTL, and suggest that the anti-CD8-resistant pCTL present in adult peripheral lymphoid tissues may require additional post-thymic maturation. We therefore analyzed the development of peripheral pCTL during ontogeny, to determine whether anti-CD8-resistant pCTL are delayed in their development, compared with conventional anti-CD8-sensitive pCTL. Figure 11 (*top panels*)

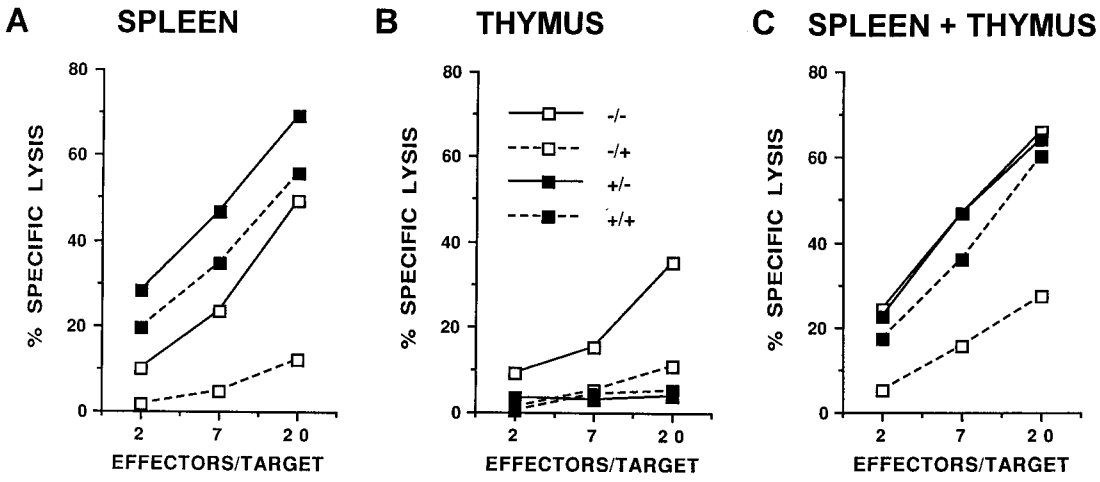

FIGURE 9. Thymus cells do not inhibit the generation of anti-CD8-resistant CTL from splenic precursors in MLC. B6 responder cells (2.5×10^6/well) from spleen or thymus, or from a mix of spleen and thymus (2.5×10^6 of each per well) were cocultured for 5 days with BALB/c splenic stimulator cells (5×10^6/well), either with (solid symbols) or without (open symbols) 83-12-5 anti-CD8α mAb. On day 5, effector cells were tested for lytic activity against P815 targets in the presence (dotted lines) or absence (solid lines) of anti-CD8 mAb.

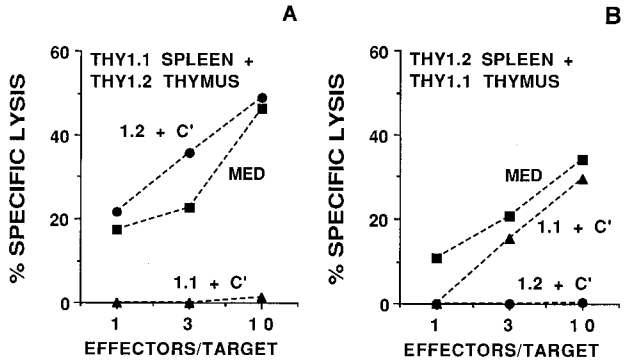

FIGURE 10. Spleen cells do not facilitate the generation of anti-CD8-resistant CTL from thymic precursors in MLC. B6/Thy-1.2 and B6.PL/Thy-1.1 splenic and thymic responder cells in the indicated combinations (2.5×10^6 of each per well) were cocultured for 5 days with bm1 splenic stimulator cells (5×10^6/well), with 53-6.72 anti-CD8α (a nonlytic, non-complement-binding) mAb. On day 5, the effector populations were depleted of either Thy-1.2-positive cells or Thy-1.1-positive cells by treatment with the appropriate anti-Thy-1 mAb and complement, or were left untreated (MED). The effectors were then tested for lytic activity against bm1 targets in the presence of anti-CD8 mAb. The combinations shown in the figure are thus all of the +/+ category; however, the −/−, −/+, and +/− combinations, as well as syngeneic responder cell mixes (B6 spleen and thymus, and B6.PL spleen and thymus) were all performed as controls, but are not shown.

depicts the bm1-specific CTL activity generated in B6 anti-bm1 MLC using splenic responders from 5-day-old mice (representative of 5- to 7-day-old mice), 15-day-old mice (representative of 13- to 23-day-old mice), and adult mice. Splenic responders from 5-day-old mice generated anti-CD8-sensitive CTL in MLC in the absence of anti-CD8 mAb, but did not generate anti-CD8-resistant CTL in MLC in the presence of anti-CD8. In experiments analogous to those shown in Figures 9 and 10, we found that spleen cells from very young mice did not suppress the generation of adult splenic anti-CD8-resistant CTL, nor did adult spleen cells rescue anti-CD8-resistant CTL activity from neonatal splenic responder cell cells (data not shown). Splenic responders from 15-day-old mice generated anti-CD8-sensitive CTL in MLC in the absence of anti-CD8 mAb, and generated low levels of anti-CD8-resistant CTL in MLC in the presence of anti-CD8 mAb. B6 mice exhibited adult levels of splenic anti-CD8-resistant pCTL/CTL by 4 to 6 wk of age (data not shown). Thus, the in vivo development of peripheral anti-CD8-resistant pCTL is much slower than that of peripheral anti-CD8-sensitive pCTL.

We also analyzed the development of thymic pCTL during ontogeny (Fig. 11, *bottom panels*). Surprisingly, we found that thymic responder populations from very young mice generated strong anti-CD8-resistant CTL responses in MLC in the presence of anti-CD8 mAb, in contrast to adult thymocyte populations. Fifteen-day-old mice had already lost their thymic anti-CD8-resistant pCTL population, and were beginning to express detectable splenic anti-CD8-resistant pCTL, as noted above. These results demonstrate that the expression of anti-CD8-resistant pCTL/CTL activity is developmentally regulated, that the control of that regulation may be quite complex, and that anti-CD8-sensitive and anti-CD8-resistant pCTL have very different tissue distributions throughout ontogeny.

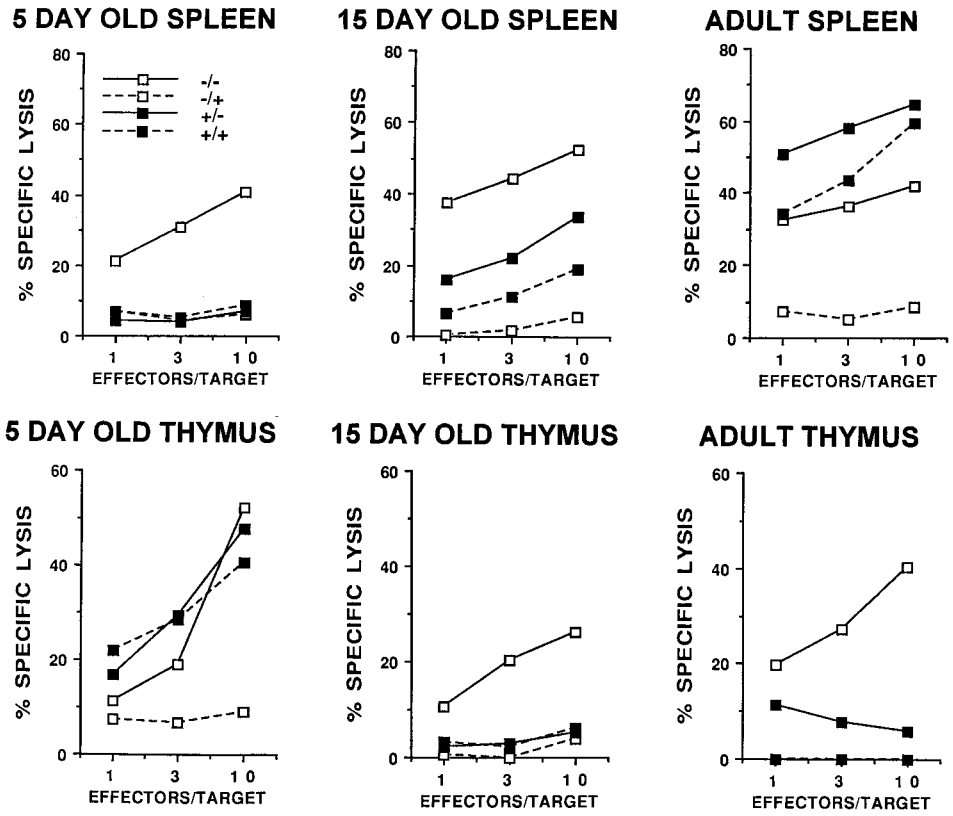

FIGURE 11. In the presence of anti-CD8 mAb, anti-CD8-resistant CTL develop in MLC from thymic, but not peripheral neonatal T cells. B6 responder cells (2.5×10^6/well) from spleen or thymus of adults or young mice were cocultured for 5 days with bm1 splenic stimulator cells (5×10^6/well), either with (solid symbols) or without (open symbols) 3.155 anti-CD8α mAb. On day 5, effector cells were tested for lytic activity against bm1 targets in the presence (dotted lines) or absence (solid lines) of anti-CD8 mAb.

Discussion

CTL lytic effector function is an important component of immune responses against allogeneic histocompatibility Ags expressed on transplanted organs and tissues, including Ags encoded within the MHC and non-MHC mHags (41–44). In addition, CTL lysis of target cells contributes importantly to immune protection against viral infections (12, 45) and tumors (46, 47). Elucidation of the requirements and mechanisms for efficient CTL development from precursor cells could therefore contribute directly to both Ag-specific tolerance and adoptive immunotherapy protocols.

In this study, we have investigated the relationship of conventional anti-CD8-sensitive CTL and a novel population of anti-CD8-resistant allospecific CTL that we had previously identified. These CTL are dependent on anti-CD8 mAb during their in vitro induction, perhaps to reduce their avidity for alloantigen to an appropriate TCR-triggering range for functional activation in in vitro cultures, in which Ag is extremely abundant (17). These effector cells, which can be induced both in vitro (17, 18) and in vivo (6, 22, 23), may play an important role in rejection of MHC class I alloantigen-bearing organ and tissue transplants (6, 22, 23). The requirements for the generation, activation, and inactivation of these cells are therefore of interest from both physiologic and clinical perspectives. Comparisons of anti-CD8-resistant CTL with conventional anti-CD8-sensitive CTL may reveal functional or developmental properties that would facilitate the intentional activation or inactivation of each of these populations in response to foreign or self Ags.

Conventional anti-CD8-sensitive CTL and the novel anti-CD8-resistant CTL populations share the phenotypic markers we have tested to date, they have similar but not identical Ag-specific repertoires, and they both appear to be generated from naive unprimed T cells. However, these CTL populations exhibit important functional differences. They differ in their kinetics of activation in vitro, their dependence on exogenous cytokines, their use of lytic effector mechanisms, and their tissue distribution during ontogeny. Based on these results, we favor the hypothesis that these CTL populations represent distinct T cell lineages or subsets, and not merely different TCR avidity ranges within a single T cell lineage or subset.

A major finding of this study is that anti-CD8-resistant effector cells have a unique developmental pattern, in that they can be generated only from the thymus of young mice, and only from the peripheral tissues of adult mice. This developmental pattern does not appear to reflect either a suppressive influence or the lack of a required helper or accessory cell in the young peripheral tissues and adult thymus. Rather, this pattern suggests that anti-CD8-resistant CTL have a different developmental program than conventional anti-CD8-sensitive CTL, which are readily generated from both thymus and peripheral tissues in both young and adult mice.

These novel anti-CD8-resistant pCTL/CTL (the +/+ group in our figures, whose precursor frequencies we had shown previously are comparable with those of conventional anti-CD8-sensitive CTL; Ref. 17) also have a different developmental pattern than the few CD8-independent CTL that are generated in the absence of anti-CD8 mAb in culture (the −/+ group in our figures), which appear to be equally weak in the thymus and peripheral tissues of neonatal and adult mice (Fig. 11). Such rare $CD8^-$-independent CTL responses may be enriched in mice treated in vivo with mAb specific for the α_3 domain of MHC class I to block $CD8/\alpha_3$ interactions required by conventional $CD8^-$-dependent CTL (48), although we did not detect such enrichment in in vitro cultures treated with bivalent anti-CD8 mAb (17), which should also have blocked $CD8/\alpha_3$ interactions. Thus, the relationship of the rare $CD8^-$-independent CTL to the relatively frequent anti-CD8-resistant CTL that we have studied in vitro and others have studied in vivo (6) is still unclear, although the tissue distribution differences noted above are most compatible with their being distinct lineages or maturation stages.

The relationship between the anti-CD8-resistant CTL in the neonatal thymus and in the adult peripheral tissues is not yet known. One possible scenario is that neonatal thymic anti-CD8-resistant pCTL emigrate to the periphery as a cohort, leaving the adult thymus deficient in this T cell functional population. Cohort "waves" of distinct subsets of T cells through the neonatal thymus have been reported (49, 50), although the control of this development and emigration is not fully understood.

A second possible scenario is that the neonatal thymic anti-CD8-resistant pCTL population has a limited life span, and may be distinct from the adult peripheral anti-CD8-resistant pCTL population. In that case, adult peripheral anti-CD8-resistant pCTL/CTL may represent either an extrathymic T cell lineage or a post-thymic maturation stage. Athymic nude mice develop some $CD8^+$ peripheral CTL (51–53), and some peripheral CTL in thymus-engrafted nude mice appear to have developed extrathymically (54, 55). Intraepithelial T cells also include an extrathymic population (24, 25), so an extrathymic origin for the anti-CD8-resistant CTL we have analyzed from normal mice would not be unprecedented. Some IEL are thymus derived, but may require a further thymic influence to complete their maturation process in the periphery (27); this scenario may also apply to the peripheral anti-CD8-resistant CTL we have studied. A subset of T cells found in the liver also has unusual phenotypic characteristics and may develop in situ, in the absence of a thymic influence (28, 29). Finally, the mature T cells in the periphery after thymic involution may be replenished from extrathymic sources, or they may simply be very long-lived.

The functional relationship between anti-CD8-resistant CTL and anti-CD8-sensitive CTL is not yet clear. It is clear that both populations can be primed in vivo in the absence of anti-CD8 mAb, and that both populations require such priming for subsequent detection in vitro in response to non-MHC alloantigens (Fig. 3). Berzofsky and colleagues have similarly demonstrated that both anti-CD8-sensitive and anti-CD8-resistant antiviral CTL can and must be primed in vivo (19). These results indicate that anti-CD8-resistant CTL are unlikely to represent memory cells resulting from a previous unintentional cross-reactive Ag exposure. However, the APC requirements, costimulation requirements, Th cell dependence, and tolerance/anergy susceptibility of the novel anti-CD8-resistant CTL population in vivo are unknown. Similarly, the functional activities, other than target cell lysis, of anti-CD8-resistant CTL are unknown. $CD8^+$ T cells can be assigned to provisional subsets based on the spectrum of cytokines they secrete (56, 57), but the cytokines produced by anti-CD8-resistant CTL and anti-CD8-sensitive CTL remain to be extensively characterized.

In spite of the many questions that remain to be answered regarding the relationship of anti-CD8-sensitive and anti-CD8-resistant CTL, the ability to selectively induce or restimulate high potency anti-CD8-resistant CTL in vitro, by inclusion of anti-CD8 mAb in culture, raises the possibility that these effector cells could be useful in immunotherapy protocols. The use of anti-CD8 mAb to reduce avidity for Ag and therefore reduce the strength of activation, to allow productive stimulation of very high avidity T cells, does not require knowledge of the precise antigenic peptide recognized by the T cells. The utility of this strategy thus applies

to situations in which the parallel strategy of careful Ag dose titration cannot be used because the Ag has not yet been identified or purified, as may be the case for many tumor and viral Ags. Since anti-CD8-resistant CTL appear to be much less dependent than anti-CD8-sensitive CTL on target cell expression of Fas, anti-CD8-resistant CTL might be expected to kill a broader range of target cells; this may also be particularly relevant for immunotherapy directed against Fas^- tumor cells or virally infected cells.

The potential use of anti-CD8-resistant CTL in antitumor or antiviral immunotherapy requires that these cells express a TCR repertoire against tumor or viral Ags. The relationship of allospecific CTL and Ag-specific CTL is therefore an important issue for such studies. Self MHC class I-restricted CTL responses to nominal intracellular Ags, such as tumor or viral Ags, require Ag degradation to peptides by proteosomes, transport to the endoplasmic reticulum by transporter complexes involving the TAP-1 and TAP-2 proteins, association with MHC class I heavy chain and β_2-microglobulin, and transport to the cell membrane for recognition by CTL. While we have not yet examined self MHC class I-restricted CTL responses to tumor or viral Ags, we have generated anti-CD8-resistant self MHC class I-restricted CTL responses to mHags (Fig. 3). These Ags are polymorphic cell protein Ags that undergo all of the same intracellular processing and presentation steps as tumor or viral protein Ags. Like CTL responses to tumor or viral Ags, CTL responses to mHags require in vivo priming, are $CD8^+$, and utilize the TCR-$\alpha\beta$ repertoire. We therefore expect that our results with mHag-specific CTL will also apply to tumor-specific and virus-specific CTL induction protocols.

In conclusion, we have presented evidence that anti-CD8-resistant pCTL and CTL may represent a new functional T cell subset or lineage. The origin and developmental program of anti-CD8-resistant pCTL/CTL remain to be defined, and should contribute to an understanding of how these T cells could be intentionally activated or inactivated in treatment of cancer, transplant rejection, autoimmune diseases, and immunodeficiency diseases.

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