

DIFFERENTIATION OF Ia-REACTIVE CD8<sup>+</sup> MURINE  
T CELLS DOES NOT REQUIRE Ia ENGAGEMENT  
Implications for the Role of CD4 and CD8 Accessory Molecules  
in T Cell Differentiation

BY TOSHIAKI MIZUOCHI,\* LUCIO TENTORI,† SUSAN O. SHARROW,\*  
ADA M. KRUISBEEK,† AND ALFRED SINGER\*

*From the \*Immunology Branch and †Biological Response Modifiers Program, National Cancer  
Institute, National Institutes of Health, Bethesda, Maryland, 20892*

A necessary step in the differentiation of stem cells into functionally competent T cells involves selection of precursor cells based on their receptor specificity for thymic MHC-encoded determinants (1). In support of this concept is the observation that MHC class II-restricted CD4<sup>+</sup> T cells fail to appear in mice whose Ia determinants are blocked by anti-Ia mAbs administered in vivo from birth onward (2). However, CD4<sup>+</sup> T cells are not the only cells reactive to MHC class II determinants, as there exists a CD8<sup>+</sup> T cell subset that is also reactive to MHC class II determinants (3, 4). This CD8<sup>+</sup> class II-reactive CTL subset represents the primary exception to the correlation of CD4 expression with MHC class II recognition and CD8 expression with MHC class I recognition (5). The present study was undertaken to determine if the differentiation of CD8<sup>+</sup> class II-reactive T cells, like that of CD4<sup>+</sup> T cells, required engagement of MHC class II determinants during development. We addressed this issue in mice chronically administered anti-Ia mAb where the differentiation of CD4<sup>+</sup> class II-specific T cells was arrested. We found that, in contrast to its arrest of CD4<sup>+</sup> T cell differentiation, in vivo anti-Ia blockade did not demonstrably interfere with the differentiation of Ia-reactive CD8<sup>+</sup> T cells.

**Materials and Methods**

*Animals.* C57BL/6 (B6) and C57BL/10 (B10) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. B6.C-H-2<sup>bm1</sup> (bm1) and B6.C-H-2<sup>bm12</sup> (bm12) mice were bred in our own animal colony.

*Monoclonal Antibodies.* Anti-I-A<sup>b</sup> mAb was purified Ig from ascites of either the Y-3P or M5/114 hybridoma cell lines obtained from the American Type Culture Collection, Rockville, MD. Anti-CD4 mAb was a culture supernatant of the anti-L3T4 hybridomas cell line GK1.5 (6). Anti-CD8 mAb was a culture supernatant of the anti-Lyt-2.2 hybridoma cell line 83-12-5 produced by Dr. J. Bluestone, NIH.

*In Vivo Anti-Ia Treatment.* Neonatal B6 mice were treated within 24 h of birth with purified anti-I-A<sup>b</sup> mAb prepared from ascites from either hybridoma Y-3P or M5/114. Intraperitoneal injections of 200 µg, 400 µg, and 600 µg of antibody were given daily during the first, second, and third week of life, respectively. Saline-injected littermates were used as control animals.

Address correspondence to A. Singer, Immunology Branch, NIH, Bldg. 10, Room 4B-17, Bethesda, MD 20892.

*In Vitro Generation of CTL.* CTL were generated in 2-ml cultures consisting of  $4 \times 10^6$  responder spleen cells and  $4 \times 10^6$  2,000 rad irradiated stimulator spleen cells in RPMI culture medium supplemented with the 18-h supernatant from Con A-induced spleen cell cultures to which  $\alpha$ -methyl-mannoside had been added (ConASN) (4). On day 5, graded numbers of effector cells were added to  $4 \times 10^3$   $^{51}\text{Cr}$ -labeled LPS-induced spleen target cells.

*Flow Cytometry.* Two- and three-color immunofluorescence analysis of spleen cells and thymocytes from saline-treated control and anti-I-A<sup>b</sup> mAb-treated mice was performed using a modified Becton Dickinson & Co. (Mountain View, CA) Dual Laser FACS II as described (2).

### Results and Discussion

B6 mice were administered anti-I-A<sup>b</sup> mAb by daily injections beginning at birth and continuing for up to 4 wk. Treated animals were assessed at 2-4 wk of age, with saline-injected littermates serving as control animals. The effect of anti-I-A<sup>b</sup> mAb administration on the appearance of mature CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the thymus and spleens of the five experimental animal groups used in the present study was assessed by two-color immunofluorescence and flow cytometry (Table I). Consistent with previous reports (2), both the thymus and spleen of anti-I-A<sup>b</sup>-treated B6 mice were profoundly depleted of single-positive CD4<sup>+</sup> CD8<sup>-</sup> T cells, but contained single-positive CD8<sup>+</sup> CD4<sup>-</sup> T cells in nearly normal numbers (Table I). The experimental animals used in the present study were also depleted of mature CD4<sup>+</sup> T cells by functional criteria, as indicated by their ability to secrete IL-2 only in response to stimulator cells expressing MHC class I (K<sup>bm1</sup>) but not MHC class II (I-A<sup>bm12</sup>) alloantigens (data not shown). Thus, by both physical and functional assessments, the anti-I-A<sup>b</sup> treated H-2<sup>b</sup> mice used in the present study were depleted of mature CD4<sup>+</sup> T cells.

To assess the effect of anti-Ia treatment on the differentiation of CD8<sup>+</sup> Ia-reactive CTL, unprimed cell populations from control and anti-I-A<sup>b</sup>-treated mice were stimulated in vitro with MHC class II (I-A<sup>bm12</sup>) disparate stimulators, and the cultures assayed on day 5 for the generation of class II-allospecific CTL by their ability to lyse LPS-induced blast target cells (Fig. 1). Anti-I-A<sup>b</sup>-treated mice did contain class II-allospecific CTL precursors (pCTL) as demonstrated by their ability to generate anti-I-A<sup>bm12</sup> class II allospecific CTL (Fig. 1, *a* and *b*) whose lysis of bm12 target cells was blocked by anti-Ia mAb added to the cytolytic effect assay (Fig. 1, *c* and *e*). Furthermore, the generation of class II-allospecific CTL from anti-I-A<sup>b</sup>-treated mice was blocked by the addition of anti-Ia mAb to the response cultures (Fig. 1, *c* and *e*). The antibody blockade in each case was specific because the

TABLE I  
*Phenotype of T Cells Present in H-2<sup>b</sup> Mice Injected with Anti-I-A<sup>b</sup> mAb*

In vivo treatment	CD4 <sup>+</sup> CD8 <sup>-</sup> T cells		CD8 <sup>+</sup> CD4 <sup>-</sup> T cells	
	Thymus	Spleen	Thymus	Spleen
	%		%	
Saline	11.1 ± 1.4	9.9 ± 3.4	2.7 ± 0.7	6.3 ± 1.8
αI-A <sup>b</sup>	0.8 ± 0.1	0.5 ± 0.1	2.5 ± 0.6	4.7 ± 1.4

Two-color flow cytometry analysis of cell surface CD4 and CD8 expression in thymocytes and spleen cells from five independent groups of anti-I-A<sup>b</sup>-treated and control mice. Data are expressed as mean ± SE.

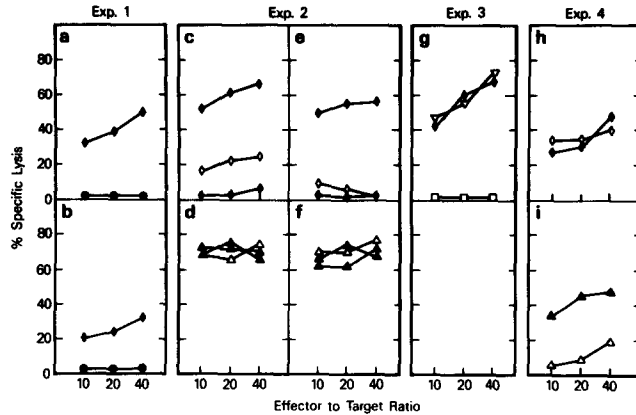


FIGURE 1. Generation of CD8<sup>+</sup> class II allospecific CTL in B6 mice treated with anti-I-A<sup>b</sup> mAb. Experimental groups are displayed in top panels, control groups are displayed in bottom panels. Exp. 1: Spleen cells from B6 mice chronically injected with either anti-I-A<sup>b</sup> (a) or saline (b) and cultured in the presence of ConASN for 5 d with bm12 stimulator spleen cells were tested on LPS-induced target cells from bm12 (◆) or B6 (●). Exp. 2: Experimental thymocytes (c) or experimental splenocytes (e) from anti-I-A<sup>b</sup>-treated B6 animals were cultured

in the presence of ConASN for 5 d with bm12 stimulator cells and assayed on LPS-induced bm12 (◆) target cells. Control thymocytes (d) or control splenocytes (f) from saline-injected B6 animals were cultured in the presence of ConASN for 5 d with bm1 stimulators and assayed on LPS-induced bm1 (▲) target cells. M5/114 mAb which reacts with both I-A<sup>b</sup> and I-A<sup>bm12</sup> determinants was added (25% vol/vol) either to the induction cultures (open symbols) or to the CTL effector assay (half-filled symbols). (Closed symbols) Responses of CTL generated in the absence of anti-I-A<sup>b</sup> mAb and assayed in the absence of anti-I-A<sup>b</sup> mAb. Exp. 3: Spleen cells from anti-I-A<sup>b</sup>-treated B6 mice were cultured with bm12 stimulators for 5 d in the presence of ConASN and assayed on bm12 LPS-induced bm12 target cells (g). The effector cells were treated just before the CTL effector assay with C' (◆), anti-CD4 mAb + C' (∇), or anti-CD8 mAb + C' (□). Exp. 4: Spleen cells from anti-I-A<sup>b</sup>-treated B6 mice (h and i) were cultured with either bm12 (h) or bm1 (i) stimulators in the presence of ConASN for 5 d and then assayed on LPS-induced blast target cells from stimulator-type bm12 (◆) or bm1 (▲) mice. The CTL effector assay was performed either in the absence (closed symbols) or in the presence of anti-CD8 blocking antibody (open symbols).

anti-Ia mAb had no effect on class I (anti-K<sup>bm1</sup>)-allospecific responses of control cell populations when added to either the induction cultures or the cytolytic effector assays (Fig. 1, d and f). The class II-allospecific CTL effectors generated from anti-I-A<sup>b</sup>-treated mice were CD8<sup>+</sup>CD4<sup>-</sup> (Fig. 1 g), but anti-CD8 mAb did not block their lysis of bm12 target cells (Fig. 1 h), a result similar to that observed for CD8<sup>+</sup> class II-allospecific CTL from normal mice (3). In contrast, the anti-CD8 mAb did block target cell lysis by anti-K<sup>bm1</sup> CTL generated from the same experimental mice (Fig. 1 i). Thus, these results demonstrate that CD8<sup>+</sup> class II-reactive precursors do differentiate in mice chronically treated with anti-Ia mAb even though the same mice are essentially devoid of mature CD4<sup>+</sup> T cells.

Since anti-I-A<sup>b</sup>-treated animals contained CD8<sup>+</sup> CTL reactive with allogeneic class II determinants, we next assessed their reactivity to self class II determinants. However, responder cells from anti-I-A<sup>b</sup>-treated mice only generated anti-I-A<sup>bm12</sup> CTL but failed to generate CTL against self I-A<sup>b</sup> (Fig. 2), a result that cannot be trivially explained by carryover of injected anti-I-A<sup>b</sup> mAb into the response cultures because carryover of the injected M5/114 mAb should also have blocked the generation of anti-I-A<sup>bm12</sup> CTL since this mAb binds to both Ia molecules (see Fig. 1). It is tempting to attribute the tolerance to self-Ia determinants of the experimental mice to the presence in vivo of small but tolerogenic amounts of unblocked Ia determinants. However, few Ia<sup>+</sup> cells are present in anti-Ia-treated mice (2), and their Ia determinants are either blocked by antibody or modulated off the cell surface

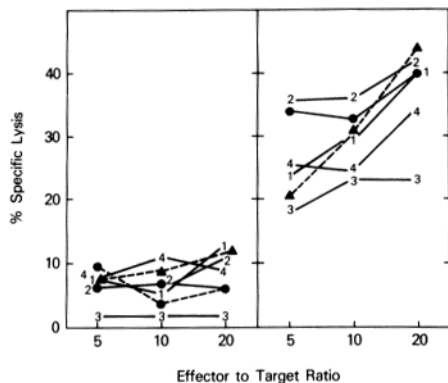


FIGURE 2. Failure to detect anti-I-A<sup>b</sup> CTL in anti-I-A<sup>b</sup>-treated B6 mice. Splenocytes (solid lines) from either four individual 3-wk-old anti-I-A<sup>b</sup> (M5/114)-treated B6 mice (1-1, 2-2, 3-3, 4-4) or from saline injected littermates (●) were cultured in vitro in the presence of ConASN with 2,000 rad irradiated spleen stimulator cells from B6 (left panel) or bm12 (right panel). In a different experiment, thymocytes (dashed lines) from either anti-I-A<sup>b</sup> (M5/114)-treated B6 mice (▲) or saline-injected littermates (●) were cultured in vitro in the presence of ConASN with 2,000 rad irradiated spleen stimulator cells from B6 (left panel) or bm12 (right panel). CTL effector cells were assayed on LPS-induced B6 (left panel) or bm12 (right panel) target cells at the indicated effector-to-target ratios.

(W. van Ewijk and A. M. Kruisbeek, unpublished observations). It is conceivable, but unlikely, that CD8<sup>+</sup> T cell tolerance to I-A<sup>b</sup> determinants results from an anti-(anti-I-A<sup>b</sup>) idiotype-(antiidiotype) regulatory network mediated by CD8<sup>+</sup> T cells and initiated by in vivo administration of anti-I-A<sup>b</sup> mAb. Whatever the mechanism, these data indicate that anti-Ia blockade does not interfere with induction of self-Ia tolerance in CD8<sup>+</sup> Ia-specific T cells.

Finally, the striking contrast in the effect of anti-I-A<sup>b</sup> antibody treatment on the differentiation of CD4<sup>+</sup> and CD8<sup>+</sup> Ia-reactive T cells made us question if developing CD4<sup>+</sup>CD8<sup>-</sup> thymocytes might somehow be unique targets for direct elimination by the injected anti-Ia mAb. Because murine thymocytes do passively acquire Ia determinants that could potentially serve to bind anti-Ia mAb to their cell surfaces (7), we examined expression of cell surface Ia determinants by distinct thymocyte subpopulations using three-color flow cytometry (Table II). Cell surface Ia expression was detected on only a minority of CD4<sup>+</sup>CD8<sup>-</sup> thymocytes, and was no greater than that found on CD4<sup>-</sup>CD8<sup>+</sup> thymocytes (Table II). These results argue against the possibility that CD4<sup>+</sup> T cells are unique targets for direct elimination by circulating anti-Ia antibodies. Instead, the selective elimination of CD4<sup>+</sup>CD8<sup>-</sup> T cell differentiation by anti-I-A<sup>b</sup> mAb treatment appears to be an indirect consequence of blocking I-A<sup>b</sup> determinants that are necessary for the differentiation of CD4<sup>+</sup> T cells but that are not necessary for the differentiation of CD8<sup>+</sup> T cells, even those reactive with Ia allodeterminants.

The present study demonstrates that differentiation of Ia-reactive CD8<sup>+</sup> T cells does not require engagement of MHC class II determinants, indicating that their differentiation either does not involve MHC engagement at all, or involves engagement of MHC class I determinants. The most straightforward interpretation of these results is that Ia-reactive CD8<sup>+</sup> T cells are conventional CD8<sup>+</sup> T cells that are selected during differentiation by self class I MHC determinants, but whose receptors fortuitously crossreact on MHC class II alloantigens with sufficiently high affinity to lyse allo-Ia-bearing target cells in the absence of either CD4 or CD8 affinity contributions. In fact, a few CD8<sup>+</sup> CTL clones have been described recently (8, 9) that do cross reactively recognize both class I and class II MHC allodeterminants, and whose lysis of allo-Ia-bearing targets is independent of CD4 and CD8 affinity contributions.

TABLE II  
*Cell Surface Expression of Ia Determinants on B10 Thymocytes*

Whole thymocytes	Percent Ia <sup>+</sup> cells			
	Thymocyte subpopulations			
	CD4 <sup>-</sup> 8 <sup>-</sup>	CD4 <sup>+</sup> 8 <sup>+</sup>	CD4 <sup>+</sup> 8 <sup>-</sup>	CD4 <sup>-</sup> 8 <sup>+</sup>
34.0	27.2	34.4	29.4	41.4

Results of three-color flow cytometry in which each cell was simultaneously assessed for CD4, CD8, and Ia expression. Percentage of Ia<sup>+</sup> cells reflects number of cells staining positively with anti-I-A<sup>b</sup> mAb minus low level nonspecific background staining by an irrelevant anti-I-A<sup>d</sup> mAb.

If current concepts are correct that thymic selection results from low affinity interactions between developing T cell precursors and self-MHC, CD4/CD8 affinity contributions to MHC binding should be unnecessary for thymic differentiation because receptor binding per se should fulfill the low affinity requirements for thymic differentiation. Yet, the present study has observed that differentiation of precursors into CD4<sup>+</sup> T cells strictly requires engagement of Ia, whereas differentiation of precursors into CD8<sup>+</sup> T cells appears completely independent of Ia, regardless of the receptor specificities they express. Consequently, we would like to suggest that the primary role of CD4 and CD8 molecules in T cell differentiation is not to supplement MHC binding affinity, but rather is to generate signals that are necessary to promote the differentiation of T cell precursors into mature T cells. We suggest that CD4 engagement of MHC class II molecules crosslinks CD4 molecules together (McCarthy, S. A., E. Kaldjian, and A. Singer, submitted for publication) or to MHC-specific antigen receptor molecules (10), generating activation signals that are strictly necessary for the differentiation of precursors into mature CD4<sup>+</sup> T cells. Similarly, CD8 engagement of MHC class I molecules crosslinks CD8 molecules together (McCarthy, S. A., et al., submitted for publication) or to MHC-specific antigen receptor molecules (10), generating activation signals that are strictly necessary for the differentiation of precursors into mature CD8<sup>+</sup> T cells. Thus, CD4<sup>+</sup> T cell differentiation would strictly require Ia engagement and CD8<sup>+</sup> T cell differentiation would strictly require class I engagement, regardless of the specificity of their antigen receptors.

In conclusion, the present study demonstrates that unlike CD4<sup>+</sup> Ia-reactive T cells, differentiation of CD8<sup>+</sup> Ia-reactive T cells does not require Ia engagement. In our opinion, the present study can best be understood by an MHC-dependent signaling role for CD4 and CD8 molecules in promoting T cell differentiation, and that anti-Ia blockade selectively arrests the differentiation of CD4<sup>+</sup> T cells, not only because it interferes with receptor-Ia interactions, but, more importantly, because it interferes with CD4-Ia interactions.

### Summary

The present study was undertaken to assess the Ia differentiation requirements of CD8<sup>+</sup> class II-allospecific CTL, whose CD8<sup>+</sup> phenotype is apparently "discordant" with their MHC class II reactivity. To do so, we compared the effect of in vivo anti-Ia blockade on the differentiation of Ia-reactive CD8<sup>+</sup> CTL with its effect

on the differentiation of CD4<sup>+</sup> T cells. We found that anti-Ia blockade did not detectably interfere with the differentiation of CD8<sup>+</sup> Ia-reactive CTL, even though it arrested the differentiation of CD4<sup>+</sup> T cells. Thus, the differentiation of CD4<sup>+</sup> T cells is strictly dependent upon Ia engagement, whereas the differentiation of CD8<sup>+</sup> T cells, even those with reactivity against MHC class II alloantigens, does not require Ia engagement. These results support the concept that Ia-reactive CD8<sup>+</sup> T cells are conventional CD8<sup>+</sup> CTL, probably selected by self-class I MHC molecules during differentiation, whose receptors fortuitously crossreact on MHC class II alloantigens. Taken together, the present data indicate an intimate relationship between CD4/CD8 expression with MHC class specificity during T cell differentiation and selection. We suggest that an active triggering role for CD4 and CD8 accessory molecules in T cell differentiation is best able to explain these observations.

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### References

1. Fink, P. J., and M. J. Bevan. 1978. H-2 antigens of the thymus determine lymphocyte specificity. *J. Exp. Med.* 148:766.
2. Kruisbeek, A. M., J. J. Mond, B. J. Fowlkes, J. A. Carmen, S. Bridges, and D. Longo. 1985. Absence of the Lyt2<sup>-</sup>, L3T4<sup>+</sup> lineage of T cells in mice treated neonatally with anti-I-A correlates with absence of intrathymic I-A-bearing antigen-presenting cell function. *J. Exp. Med.* 161:1029.
3. Shinohara, N., and M. Kojima. 1984. Mouse alloantibodies capable of blocking cytotoxic T cell function. V. The majority of I region-specific CTL are Lyt2<sup>+</sup> but are relatively resistant to anti-Lyt-2 blocking. *J. Immunol.* 132:578.
4. Golding, H., T. Mizuochi, S. A. McCarthy, C. A. Cleveland, and A. Singer. 1987. Relationship among function, phenotype, and specificity in primary allospecific T cell populations. Identification of phenotypically identical but functionally distinct primary T cell subsets that differ in their recognition of MHC class I and class II determinants. *J. Immunol.* 138:10.
5. Swain, S. L. 1983. T cell subsets and the recognition of MHC class. *Immunol. Rev.* 74:129.
6. Dialynas, D. P., Z. S. Quan, K. A. Wall, A. Pierres, J. Quintans, M. R. Loken, M. Pierres, and F. W. Fitch. 1983. Characterization of the murine T cell surface molecule designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu3/T4 molecule. *J. Immunol.* 131:2445.
7. Sharrow, S. O., B. J. Mathieson, and A. Singer. 1981. Cell surface appearance of unexpected host MHC determinants on thymocytes from radiation bone marrow chimeras. *J. Immunol.* 126:1327.
8. Schilham, M. W., R. Lang, R. Benner, R. M. Zinkernagel, and H. Hengartner. 1986. Characterization of an Lyt2<sup>+</sup> alloreactive cytotoxic T cell clone specific for H-2D<sup>b</sup> that cross-reacts with I-E<sup>k</sup>. *J. Immunol.* 137:2748.
9. Aparicio, P., D. Jaraquemada, and J. A. Lopez de Castro. 1987. Alloreactive cytolytic T cell clones with dual recognition of HLA-B27 and HLA-DR2 antigens. Selective involvement of CD8 in the class I-directed cytotoxicity. *J. Exp. Med.* 165:428.
10. Emmrich, F., U. Strittmatter, and K. Eichmann. 1986. Synergism in the activation of human CD8 T cells by cross-linking the T-cell receptor complex with the CD8 differentiation antigen. *Proc. Natl. Acad. Sci. USA.* 83:8298.