

## **CD8<sup>+</sup> T Cell Response to Mls-1<sup>a</sup> Determinants Involves Major Histocompatibility Complex Class II Molecules**

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### **Summary**

Recent studies indicate that both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes proliferate in vitro in response to Mls-1<sup>a</sup>-encoded determinants. Using both immunogenetic and antibody blocking approaches we show here that Mls-1<sup>a</sup> responses of both subsets require expression of major histocompatibility complex (MHC) class II molecules (I-A and/or I-E) by the stimulator cells. Furthermore, CD8<sup>+</sup> T cell responses to Mls-1<sup>a</sup>/class II MHC do not require (and are in fact inhibited by) the presence of functional CD8 molecules. Taken together, our data underscore the dramatic differences between CD8<sup>+</sup> T cell responses to conventional peptide antigens as opposed to "superantigens" such as Mls-1<sup>a</sup>.

The gene product(s) of the minor lymphocyte stimulatory locus Mls-1<sup>a</sup> are poorly characterized T cell-activating determinants (1, 2). T cell recognition of Mls-1<sup>a</sup> correlates with the use of particular V region segments of the TCR  $\beta$  chain ( $V_{\beta 6}$ ,  $V_{\beta 8.1}$ , and  $V_{\beta 9}$ ), and mature T cells of both CD4<sup>+</sup> and CD8<sup>+</sup> subsets bearing these  $V_{\beta}$  are clonally deleted in Mls-1<sup>a</sup> strains (3-5). Although T cell responses to Mls-1<sup>a</sup> were originally believed to be restricted to CD4<sup>+</sup> T cells (6, 7), we and others have recently shown that CD8<sup>+</sup> as well as CD4<sup>+</sup> T cells, bearing  $V_{\beta 6}$  or  $V_{\beta 8.1}$  TCRs, respond clonally to Mls-1<sup>a</sup> determinants (8-10). In mature T cells a strong correlation exists between the expression of either the CD4 or CD8 surface molecules and the ability of TCRs to recognize antigenic peptides associated with either class II or class I MHC molecules, respectively (11). Therefore, it was interesting to investigate whether CD8<sup>+</sup> T cells (with presumably MHC class I-restricted TCRs) interact with Mls-1<sup>a</sup> in association with MHC class II molecules. As CD4 and CD8 molecules play a role in TCR-mediated recognition of conventional antigens (12), we have also explored the possible involvement of CD8 molecules in Mls-1<sup>a</sup> recognition.

### **Materials and Methods**

**Mice.** Congenic BALB/c and BALB.D2.Mls<sup>a</sup> mice were maintained from breeding pairs originally provided by Dr. H. Festenstein (London Hospital Medical College, London, UK) (13). DBA/1, B10.AQR, B10.T.6R, and B10.G mice were obtained from Harlan Olac Ltd. (Bicester, UK). F<sub>1</sub> hybrid mice were bred locally.

**Mixed Leukocyte Cultures.** Nylon wool-purified responder

splenic T cells ( $1.5 \times 10^6$ ) were cultured with irradiated (1,000 rad) anti-Thy-1 plus complement-depleted splenic stimulator cells ( $4.5 \times 10^6$ ) in 2 ml DME supplemented with 5% FCS and  $5 \times 10^{-5}$  M 2-ME. In the inhibition experiments, we used  $2.5 \times 10^6$  stimulator cells. This was the lowest number that induced specific proliferation of responder cells. In some experiments, responder populations were depleted of CD4<sup>+</sup> or CD8<sup>+</sup> cells by further treatment with rat IgM mAbs RL172.4 (anti-CD4) or 3.168.1 (anti-CD8) plus rabbit complement before culture. In the case of CD4 depletion, the resulting CD8<sup>+</sup> responder cells were further supplemented with supernatant of PMA-stimulated EL4-6.1 cells (corresponding to 30 U/ml IL-2).

**Blast Purification.** After 3 d in culture, responding T cell blasts were isolated on a Percoll density gradient and resuspended ( $2 \times 10^5$  viable cells/ml) in fresh medium supplemented with human rIL-2 (60 ng/ml). After 2-3 d, during which cell density increased 7-10-fold, cells were recovered and analyzed for  $V_{\beta}$  expression.

**Inhibition Experiments.** mAbs used for inhibition studies were 14-4-4s (anti-I-E<sup>d, k, p, r</sup>), 27-9-17 (anti-I-A<sup>d, b</sup>), and 53-6.7 (anti-CD8 $\alpha$ ). In cultures in which anti-MHC class II mAbs were used, they were added together with the stimulator spleen cells at least 30 min before the addition of responder cells, and were left throughout the culture period. Similarly, anti-CD8 mAbs were added together with the CD8<sup>+</sup> responder cells before the addition of stimulators. After 5 d, responding blast cells were isolated on a Percoll gradient and analyzed for  $V_{\beta}$  expression.

**Flow Microfluorometry.** All procedures and reagents have been described (3, 8). Cells were initially stained with TCR  $V_{\beta}$ -specific mAbs 44-22-1 (anti- $V_{\beta 6}$ ) and F23.2 (anti- $V_{\beta 8.2}$ ) followed by appropriate fluoresceinated anti-Ig. Additional staining was usually carried out with PE-conjugated GK-1.5 (anti-CD4) or biotinylated 53-6.7 (anti-CD8 $\alpha$ , revealed with avidin-PE). In experiments where mAb 53-6.7 (anti-CD8 $\alpha$ ) was used as an inhibitor, biotinylated H35-17.2 (anti-CD8 $\beta$ , revealed with avidin-PE) was used in order to avoid steric blocking.

## Results and Discussion

We have used both genetic and mAb inhibition approaches to study the activation of CD8<sup>+</sup> T cells by Mls-1<sup>a</sup> determinants and, in particular, to ask if an interaction with class II MHC molecules on the APC is necessary. Previously (8), we observed that responses of both CD4<sup>+</sup> V $\beta$ 6<sup>+</sup> and CD8<sup>+</sup> V $\beta$ 6<sup>+</sup> cells to Mls-1<sup>a</sup>, in vitro, were dependent upon the MHC of the stimulator cells, with I-E<sup>+</sup> (H-2<sup>d</sup> or H-2<sup>k</sup>) alleles being much more stimulatory than I-E<sup>-</sup> (H-2<sup>q</sup>). However, these results were difficult to interpret because there were other genetic differences (including MHC class I) between the Mls-1<sup>a</sup>-bearing stimulatory cells.

To overcome this problem, we have used stimulator cells from congenic F<sub>1</sub> mice expressing Mls-1<sup>a</sup> and differing in MHC class II alleles (I-A<sup>q/k</sup>, I-E<sup>k</sup> vs. I-A<sup>q/q</sup>, I-E<sup>-</sup>) but with the same MHC class I expression (K<sup>q/q</sup> and D<sup>d/q</sup>). By using purified responder T cells from MHC-compatible F<sub>1</sub> mice of Mls-1<sup>b</sup> genotype, we could ask formally whether the expression of appropriate MHC class II molecules by Mls-1<sup>a</sup> stimulators is necessary to activate CD8<sup>+</sup> T cells. A repre-

sentative experiment is shown in Table 1. It can be seen that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressing V $\beta$ 6 TCR were strongly enriched (greater than or equal to fourfold) in response to Mls-1<sup>a</sup> associated with I-A<sup>q/k</sup>, I-E<sup>k</sup> molecules. In contrast, when Mls-1<sup>a</sup> was associated with I-A<sup>q</sup> molecules, only a minor enrichment (less than twofold) was obtained for CD4<sup>+</sup> V $\beta$ 6<sup>+</sup> cells, and no enrichment could be detected for CD8<sup>+</sup> V $\beta$ 6<sup>+</sup> cells. Enrichment of V $\beta$ 6<sup>+</sup> cells was strictly specific for Mls-1<sup>a</sup> because it did not occur when stimulator cells from congenic Mls-1<sup>b</sup> mice expressing the same MHC class II molecules were used (data not shown). Furthermore, the high proportion of CD4<sup>+</sup> V $\beta$ 6<sup>+</sup> or CD8<sup>+</sup> V $\beta$ 6<sup>+</sup> cells responding to Mls-1<sup>a</sup> in association with I-A<sup>q/k</sup>, I-E<sup>k</sup> molecules was seen irrespective of the presence or absence of allogeneic MHC class II differences between responder and stimulator (Table 1). As an internal control, we measured the proportion of T cells expressing V $\beta$ 8.2 TCR, which is known not to be specific for Mls-1<sup>a</sup>. Stimulation by Mls-1<sup>a</sup> on an appropriate (I-E<sup>+</sup>) genetic background resulted in a

**Table 1.** Expression of TCR V $\beta$  Domains by CD4<sup>+</sup> and CD8<sup>+</sup> T Cell Blasts Responding to Mls-1<sup>a</sup> Determinants In Vitro

Responder cells (Mls-1 <sup>b</sup> )				Stimulator cells (Mls-1 <sup>a/b</sup> )				Proportion of CD4 <sup>+</sup>			Proportion of CD8 <sup>+</sup>		
K	I-A	I-E	D	K	I-A	I-E	D	Percent CD4 <sup>+</sup>	V $\beta$ 6	V $\beta$ 8.2	Percent CD8 <sup>+</sup> *	V $\beta$ 6	V $\beta$ 8.2
q/d	q/d	-/d	q/d	None				41	14.1	21.4	26	14	14.3
				q/q	q/k	-/k	q/d	68.7	53.5	5.3	73.4	52.8	8.9
				q/q	q/q	-/-	q/d	27.5	24.4	27.3	64.7	12.2	20.5
q/q	q/k	-/k	q/d	None				34.2	8.5	12.3	21.5	11.9	10.6
				q/q	q/k	-/k	q/d	68.4	55.8	2.2	72.7	45.9	2.8
				q/q	q/q	-/-	q/d	19.6	16.5	12.9	55.7	6.5	6.4

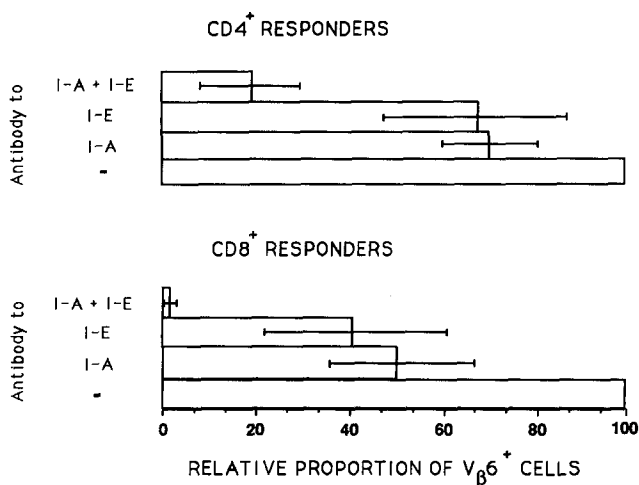
Nylon wool-purified responder T cells ( $1.5 \times 10^6$ ) from (B10.G  $\times$  BALB/c)F<sub>1</sub> or (B10.G  $\times$  B10.AQR)F<sub>1</sub> mice were mixed with irradiated (1,000 rad) T cell-depleted splenic stimulator cells ( $4.5 \times 10^6$ ) from (DBA/1  $\times$  B10.AQR)F<sub>1</sub> or (DBA/1  $\times$  B10.T.6R)F<sub>1</sub> mice. After 3 d, responding T blasts were isolated on a Percoll gradient and recultured for 2-3 d in rIL-2 (60 ng/ml). Recovered cells were double stained with mAbs directed against the indicated TCR V $\beta$  domains and either CD4 or CD8.

\* Responding T cells were CD4 depleted (CD8<sup>+</sup>) before culture. IL-2 (30 U/ml in the form of EL4 supernatant) was added from the outset.

**Table 2.** Effect of Anti-CD8 mAbs on CD8<sup>+</sup> T Cell Responses to Mls-1<sup>a</sup> Determinants

Mixed leukocyte culture			Proportion of CD8 <sup>+</sup> V $\beta$ 6 <sup>+</sup>		
Responder	Stimulator	Antibodies	Exp. 1	Exp. 2	Exp. 3
BALB/c	BALB/c	None	21.1	8	9.5
BALB/c	BALB.D2.Mls <sup>a</sup>	None	38.8	30.3	38.8
BALB/c	BALB.D2.Mls <sup>a</sup>	Anti-CD8	65.4	58.8	48.8

$1.5 \times 10^6$  nylon wool-purified splenic responder T cells (CD4 depleted) were pre-incubated with anti-Lyt-2 (CD8 $\alpha$ ) mAbs 30 min before addition of irradiated (1,000 rad) T cell-depleted splenic stimulator cells ( $2.5 \times 10^6$ ). The concentration of anti-CD8 mAb 53-6.7 used (1:200 ascites) was sufficient to completely block MHC class I-restricted recognition of peptide antigens by CD8<sup>+</sup> cytolytic T lymphocytes (15). After 5 d, T blasts were isolated on a Percoll gradient and double stained with mAbs directed against V $\beta$ 6 and Lyt-3 (CD8 $\beta$ ). Data are presented as percent CD8<sup>+</sup>V $\beta$ 6<sup>+</sup> cells after subtraction of background staining with the fluorescent conjugate alone.



**Figure 1.** Anti-MHC class II mAbs block both CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses to Mls-1<sup>a</sup>. Nylon wool-purified splenic responder T cells ( $1.5 \times 10^6$ ) from BALB/c mice, either CD4 depleted (CD8<sup>+</sup>) or CD8 depleted (CD4<sup>+</sup>), were mixed with irradiated (1,000 rad) T cell-depleted splenic stimulator cells ( $2.5 \times 10^6$ ) from BALB.D2.Mls<sup>a</sup> mice, in the absence or presence of the indicated anti-class II mAbs: 14-4-4s (anti-I-E) and/or 27-9-17 (anti-I-A). The antibody concentrations used (1:4 culture SN for anti-I-E and 1:200 ascites for anti-I-A) were determined to be functionally saturating in titration experiments (not shown). After 5 d, responding T blasts were purified and double stained for V $\beta$ 6 and CD4 or CD8. Results (mean  $\pm$  SD of five experiments) are expressed as the percentage of V $\beta$ 6<sup>+</sup> (Mls-1<sup>a</sup>-reactive) T cells relative to controls in which mAbs were omitted (44% CD4<sup>+</sup> V $\beta$ 6<sup>+</sup> and 30% CD8<sup>+</sup> V $\beta$ 6<sup>+</sup>, respectively). Background values obtained with syngeneic stimulator cells (22% CD4<sup>+</sup> V $\beta$ 6<sup>+</sup> and 10% CD8<sup>+</sup> V $\beta$ 6<sup>+</sup>) have been subtracted.

reduced proportion of V $\beta$ 8.2<sup>+</sup> T cells, presumably as a consequence of increased V $\beta$ 6 expression. These data clearly show that MHC class II molecules (I-A<sup>k</sup> and/or I-E<sup>k</sup> in these experiments) are required for Mls-1<sup>a</sup>-specific stimulation of the CD8<sup>+</sup> subset.

In parallel to the genetic approach, we have carried out antibody inhibition experiments using BALB/c (H-2<sup>d</sup>) responder T cells and congenic BALB.D2.Mls<sup>a</sup> stimulator cells. As shown in Fig. 1, when mAbs against MHC class II molecules (I-A<sup>d</sup>, I-E<sup>d</sup>, or a mixture of both) were added

in the mixed lymphocyte cultures, the response of both CD4<sup>+</sup> V $\beta$ 6<sup>+</sup> and CD8<sup>+</sup> V $\beta$ 6<sup>+</sup> subsets to Mls-1<sup>a</sup> was reduced. In the presence of anti-I-E mAbs alone, the CD4<sup>+</sup> response was lowered on average by 31%, whereas the CD8<sup>+</sup> response was reduced to a greater extent (61%). For both T cell subsets the addition of anti-I-A mAbs provoked slightly weaker inhibition (28% for CD4<sup>+</sup> vs. 50% for CD8<sup>+</sup> T cells). A mixture of both mAbs inhibited Mls-1<sup>a</sup> responses much more effectively than either one alone (80% inhibition for CD4<sup>+</sup> and 98% for CD8<sup>+</sup>). These data confirm independently that T cells of both CD4<sup>+</sup> and CD8<sup>+</sup> subsets require MHC class II molecules on the stimulator cells in order to be activated by Mls-1<sup>a</sup>. However, the degree of blocking by anti-MHC class II mAbs was more pronounced for CD8<sup>+</sup> cells than for CD4<sup>+</sup> cells, suggesting that CD8<sup>+</sup> T cells interact more weakly with Mls-1<sup>a</sup>/MHC class II than CD4<sup>+</sup> T cells.

Finally, the effect of anti-CD8 mAbs on the response of CD8<sup>+</sup> cells to Mls-1<sup>a</sup> was also investigated. For these experiments, we took advantage of the availability of mAbs directed against two different CD8 epitopes (Lyt-2/CD8 $\alpha$  and Lyt-3/CD8 $\beta$ ). mAbs against CD8 $\alpha$  were added in the mixed lymphocyte culture, whereas anti-CD8 $\beta$  mAbs were used for staining. Surprisingly, we observed a 1.5–2-fold increase in the proportion of CD8<sup>+</sup> V $\beta$ 6<sup>+</sup> cells responding to Mls-1<sup>a</sup> in the presence of anti-CD8 mAbs (Table 2). This effect could be due to the fact that blocking CD8 molecules impaired TCR/MHC class I interactions and in so doing increased the overall TCR/class II and/or TCR/Mls-1<sup>a</sup> avidity. Similar conclusions were reached recently by Kanagawa and Maki (14), who found that the introduction of CD8 (by gene transfection) into a Mls-1<sup>a</sup>-specific T-T hybridoma correlated with the loss of Mls-1<sup>a</sup> reactivity.

In conclusion, our data clearly demonstrate that CD8<sup>+</sup> T cell responses to Mls-1<sup>a</sup> involve MHC class II molecules on the APC but do not depend on (and may even be inhibited by) the presence of CD8. A similarly eccentric behavior of CD8<sup>+</sup> T cells has been found in response to Staphylococcal enterotoxin B (15), suggesting that it may represent a general property of endogenous and exogenous "superantigens".

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