

T Cell Receptor-mediated Recognition of Self-Ligand Induces Signaling in Immature Thymocytes before Negative Selection

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Summary

Shaping of the T cell repertoire by selection during intrathymic maturation involves T cell receptor (TCR) recognition of major histocompatibility complex/self-antigen complexes. In this communication, we studied the ability of minor lymphocyte stimulating (Mls) determinants to act as self-tolerogens in the selection of the T cell repertoire. We demonstrate that unprimed T cells from normal as well as TCR transgenic mice form Mls-specific conjugates with antigen-presenting cells, and that this TCR-ligand interaction leads to elevation of intercellular Ca^{2+} ($[Ca^{2+}]_i$). Peripheral T cells from TCR transgenic mice expressing receptors specific for self-Mls antigen show no reactivities to Mls^a. However, a proportion of immature thymocytes from these mice show specific binding and strong $[Ca^{2+}]_i$ elevation in response to self-antigen-presenting cells, although these thymocytes do not proliferate. This self-reactivity of thymocytes is inhibited by antibodies specific for TCR, CD4, CD8, class II molecules, lymphocyte function-associated antigen 1, and intercellular adhesion molecule 1. These results demonstrate for the first time that before thymic negative selection, immature T cells can specifically interact with cells bearing self-antigen, and suggest that the resulting TCR-dependent signal transduction events provide a basis for negative selection of self-reactive T cells.

During intrathymic maturation, immature T cells undergo positive and negative selection mediated by interactions with thymic stroma cells through receptors (TCR, CD4, and CD8) and self-ligands (MHC/self-antigen complex) (1, 2). Recent studies using TCR-specific mAb to crosslink TCR complexes indicated that TCR signaling may determine the fate of individual immature T cells (3–5). Since under physiologic conditions, T cells recognize only antigen displayed on the surface of other cells, the direct interaction of immature T cells with cells presenting self-antigens is critical to T cell development and selection. To elucidate the thymic selection mechanism, the nature of cellular interaction and resulting signal transduction was examined for the interaction between immature thymocytes and APC that express self-Mls product (6, 7).

The role of Mls determinants as self-tolerogens in the selection of the T cell repertoire has been well documented (8–12). Using Mls-specific T cell clones, we have recently established a system that permits quantitation of T cell reactivities to a given target by simultaneous measurement of T cell-APC conjugation and cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) ele-

vation (Y. Ishida et al., manuscript submitted for publication). In the present study, unprimed Mls-specific T cells from normal inbred strains as well as from mice transgenic for an Mls^a-reactive TCR (V β 8.1) (13) were studied to evaluate the TCR-dependent interaction of these T cells with their physiological ligand. In this communication, we demonstrate that parallel to a strong primary Mls-specific proliferative response, unprimed T cells from normal, as well as TCR transgenic mice, form Mls-specific conjugates with APC and increase $[Ca^{2+}]_i$. Thymocytes from TCR transgenic mice with receptor specificity for self-Mls whose peripheral T cells have no Mls^a reactivity show specific binding and substantial $[Ca^{2+}]_i$ elevation to Mls^a APC but do not proliferate. The self-Mls reactivity of thymocytes is inhibited by antibody specific for TCR, CD4, CD8, class II molecules, LFA-1, and intercellular adhesion molecule 1 (ICAM-1)¹. These results

¹ Abbreviations used in this paper: ICAM-1, intercellular adhesion molecule 1; PI, phosphatidylinositol; TG, transgenic mouse.

strongly suggest that before thymic negative selection, immature T cells intimately associate with cells bearing self-antigen through multiple ligand/receptor interactions.

Materials and Methods

Animals. AKR/J, CBA/Ca, and CBA/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C3H/HeJCR, CBA/JCR, and CBA/CaHNCr mice were obtained from the Frederick Cancer Research Facility (Frederick, MD). The generation and characterization of V β 8.1 transgenic mice (V β 8.1 TG) have been described (13). (CBA/J \times V β 8.1 TG) F_1 mice (Mls $^{a/b}$ V β 8.1 TG) were bred in our own facilities.

Reagents and Antibodies. The calcium indicator, indo-1 acetoxy-methyl pentaester (indo-1-AM), and the cationic membrane binding dye, 1,1'-didocosanyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiIC22[3]), were obtained from Molecular Probes, Inc. (Junction City, OR). 145-2C11, hamster anti-mouse CD3- ϵ (14), was generously provided by J. Bluestone (University of Chicago, Chicago, IL). F23.1, mouse anti-mouse V β 8.1,2,3 and F23.2, anti-mouse V β 8.2 (15), were kindly provided by Dr. M. Bevan (University of Washington, Seattle, WA). Goat antibody specific for mouse-IgD was generously provided by Dr. F. D. Finkelman (Uniformed Services University of the Health Sciences, Bethesda, MD). The anti-class II mAbs 10-2-16 (16), 14-4-4 (17), and anti-LFA-1 (clone FD441.8 [18]) were obtained from American Type Culture Collection (Rockville, MD). Anti-CD4 (clone GK1.5 [19]) and anti-ICAM-1 (clone YN1/1.7.4, [20]) were obtained from Dr. Ethan Shevach (National Institutes of Health, Bethesda, MD). Anti-mouse Lyt2.1 mAb was purchased from New England Nuclear (Boston, MA).

Flow Cytometry Analysis of T Cell/APC Binding and [Ca $^{2+}$] $_i$ Elevation of T Cells. Nylon nonadherent, T cell-enriched, spleen cells were loaded with 1 μ M indo-1 AM for 30 min at 37°C in serum-free medium. B cells used as APC were prepared by treating spleen cells with rabbit anti-mouse brain antiserum plus complement, and were stained by 2 μ M DiIC22(3) for 30 min at 37°C. DiIC22(3) fluorescence was stable for >24 h. After disruption of T cell/APC conjugates by antibodies to cell surface determinants, the T cells showed no DiIC22(3) staining, indicating that there is no dye transfer between conjugated cells. For the sake of enhancement of Mls expression by APC, 200 μ l of goat anti-mouse IgD antiserum was injected 24 h before mice were killed (21). 5 \times 10 5 T cells and 2 \times 10 6 APC were mixed and quickly spun to make a pellet. After 5 min at 37°C, the pellet was then gently resuspended and analyzed by a modified FACS II $^{\circ}$ (Becton Dickinson Immunocytometry System, San Jose, CA) equipped with an argon laser (2025; Spectra Physics, Mountain View, CA) and krypton laser (164-01, Spectra Physics). The krypton ion laser was operated at 100 mW at 530 nm to excite DiIC22(3) cyanine dye. For ratio measurement of indo-1, emissions were measured simultaneously at two wavelengths with a 22-nm bandpass filter centered at 485 nm (485/22), and a 25-nm bandpass filter centered at 404 nm (404/25). In measuring [Ca $^{2+}$] $_i$, the ratio of linear fluorescence at 485 nm to that at 404 nm was calculated by the data acquisition program. DiIC22(3) fluorescence was measured through a 26-nm bandpass filter centered at 575 nm. Indo-1 and DiIC22(3) signals were detected simultaneously and all parameters were first triggered by the indo-1/485 nm fluorescence signal so that only indo-1-containing cells (T cells and T cell-APC conjugates) were detected. APC-coupled T cells were identified as the indo-1 and DiIC22(3) double-positive population. Data were recorded by PDP 1/84 and MVII computers (Dig-

ital Equipment Corp., Maynard, MA) and were analyzed with program developed in our laboratory (22).

Measurement of Phosphatidylinositol (PI) Hydrolysis. Total PI hydrolysis was measured with a modification of the method of Mizuguchi et al. (23). Briefly, 10 8 T cell-enriched spleen cells were labeled with 100 μ Ci/ml [3 H]myo-inositol (Amersham Corp., Arlington Heights, IL) at 37°C for 5 h in inositol-free RPMI 1640 (Gibco Laboratories, Grand Island, NY) + 10% dialyzed FCS. The cells were washed three times to remove free radioactive inositol. T cells and B cells were resuspended at 7 \times 10 7 and 5 \times 10 6 cells/ml, respectively, in serum-free medium containing 10 mM LiCl and preincubated for 10 min. 0.1 ml T cell suspension was mixed with same amount of medium or B cell suspension. T cells were stimulated with 50 μ g/ml heterodimeric anti-CD3 ϵ chain mAb (2C11) or control antibody (2H6; hamster anti-arsenic acid). The T cell-B cell mixture was briefly centrifuged and incubated 5 min at 37°C. Cells were kept as a pellet (Exp. 1) or resuspended (Exp. 2) and incubated an additional 25 min. The reaction was stopped by the addition of a 1:2 mixture of CH $_2$ Cl/CH $_3$ OH. The lysate was extracted with a 1:1 mixture of CHCl $_3$ /H $_2$ O. The aqueous phase was applied to a 0.5-ml Dowex-1x8 (Bio-Rad Laboratories, Richmond, CA). Unbound materials were removed by extensive washing with 5 mM myo-inositol before all phosphoinositols were eluted with 1.5 ml 1.0 M Na-formate/0.1 M formic acid and counted with automatic quench correction.

T Cell Proliferation Assay. T cell proliferation was assayed as described previously (24). Varying numbers of splenic T cells or thymocytes were cultured with 5 \times 10 5 B cells inactivated by 3,000-rad irradiation. As a control, phorbol ester (PMA) and calcium ionophore (ionomycin) were added at final concentrations of 10 and 20 ng/ml, respectively.

Results

Early Signal Transduction of Unprimed T Cell to Mls Stimulation. We have recently established a system that permits quantitation of T cell reactivity to a given target by simultaneous measurement of T cell-APC conjugation and [Ca $^{2+}$] $_i$ elevation (Y. Ishida et al., manuscript submitted for publication). Using this methodology, the anti-Mls specificity of unprimed T cells from normal inbred strains as well as from mice transgenic for the Mls a reactive TCR (V β 8.1) was studied.

T cell-enriched spleen cells were loaded with indo-1 and mixed with B cells from various strains as an APC source, and T cell-APC conjugation and [Ca $^{2+}$] $_i$ were measured. Flow cytometry data of T cell interactions with selected APC are shown in Fig. 1 and the results are summarized in Table 1. T cells from Mls $^{-}$ strains, CBA/CaH and C3H/HeJ, form numerous T cell-APC conjugates that have increased [Ca $^{2+}$] $_i$ with spleen cells obtained from Mls $^{+}$ strains, AKR/J and CBA/J, whereas these T cells reveal no reactivity to nonstimulatory Mls b APC derived from CBA/CaH. This T cell-APC binding and [Ca $^{2+}$] $_i$ elevation of T cells is consistent with the high precursor frequency of Mls a -reactive T cells in splenic T cell populations. Only those T cells that bound to APC exhibited an increased [Ca $^{2+}$] $_i$, indicating that Mls a -specific T cell-APC conjugation is necessary for [Ca $^{2+}$] $_i$ elevation (Fig. 1). The Mls a -specificity of T cell-APC conjugate formation and [Ca $^{2+}$] $_i$ elevation became clearer when CBA/CaH (Mls b) V β 8.1

T cell Source:

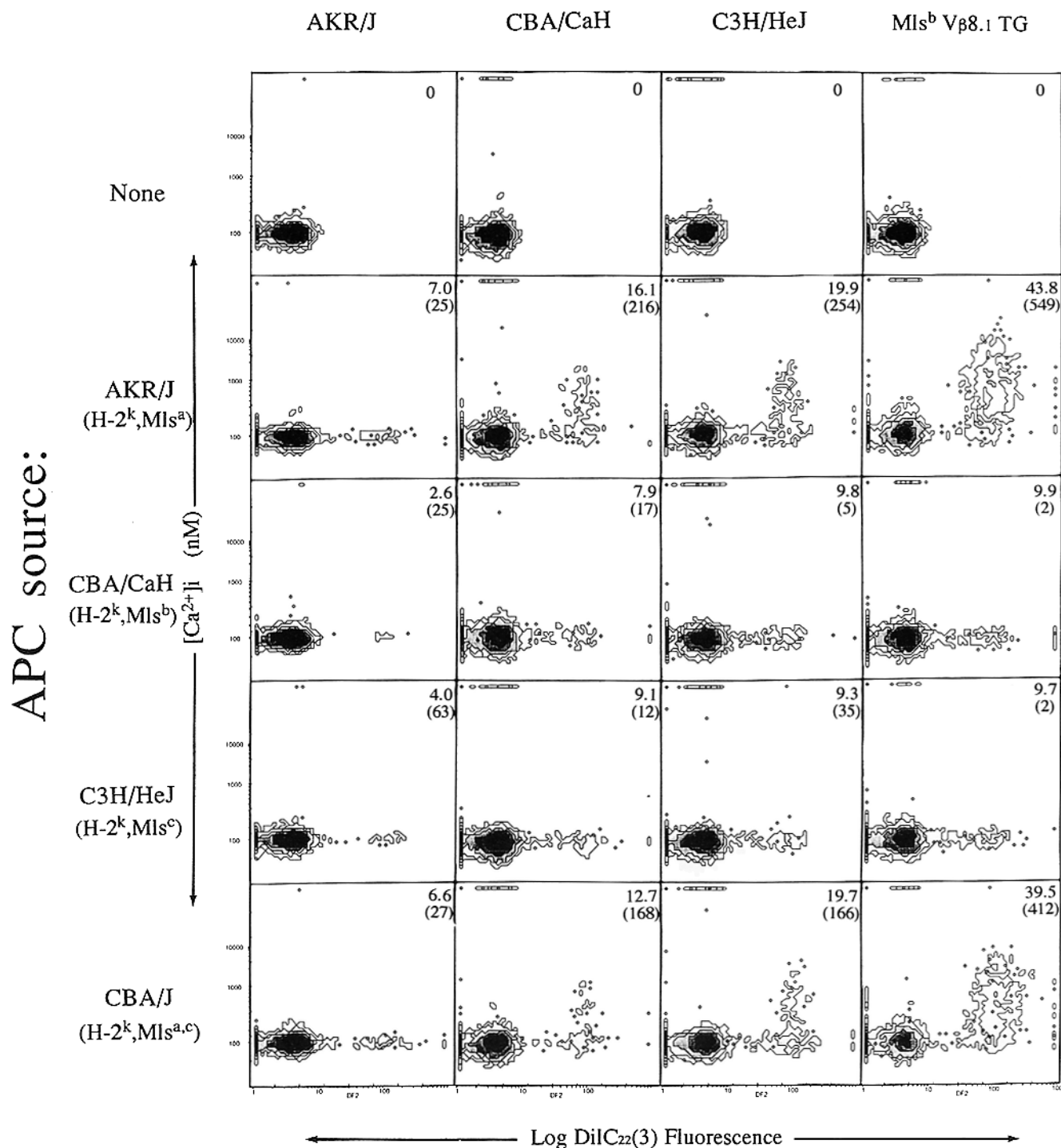


Figure 1. Flow cytometry analysis of T cell/APC binding and $[Ca^{2+}]_i$ response triggered by allogenic APC. 5×10^5 nylon wool–nonadherent, T cell–enriched, spleen cells were loaded with indo-1 AM and were mixed with 2×10^6 B cells used as APC, which were stained by DilC22(3). For the sake of enhancement of Mls expression by APC, $200 \mu\text{l}$ of goat anti–mouse IgD antiserum was injected 24 h before mice were killed. T cell/APC binding and $[Ca^{2+}]_i$ were examined by the procedure described in Materials and Methods. The x-axis indicates DilC22(3) fluorescence, which represents T cell–APC conjugates. The y-axis is the level of $[Ca^{2+}]_i$ of indo-1-loaded T cells. The indicated numbers are the percentages of T cells binding DilC22(3)-labeled APC. The number in parenthesis is the increase in $[Ca^{2+}]_i$ of APC-bound T cells above the resting level.

Table 1. *T Cell-APC Conjugation and Induction of $[Ca^{2+}]_i$ Response of Unprimed T Cells from Various H-2^k Strains*

Exp.	APC* from	Mls	T cells [†] from:							
			AKR/J		CBA/CaH		C3H/HeJ		Transgenic [§]	
			Binding	$[Ca^{2+}]_i$	Binding	$[Ca^{2+}]_i$	Binding	$[Ca^{2+}]_i$	Binding	$[Ca^{2+}]_i$
1	AKR/J	a	7.0	25	16.1	216	19.9	254	43.8	549
	CBA/J	a,c	6.6	27	12.7	168	19.7	199	39.5	412
	C58/J	a,c	6.6	156	8.1	134	15.7	166	21.8	269
	CBA/CaH	b	2.6	25	7.9	17	9.8	5	9.9	2
	C3H/HeJ	c	4.0	63	9.1	12	9.3	35	9.7	2
2	AKR/J	a	9.2	18	10.4	198	18.8	272	26.7	535
	CBA/J	a,c	12.0	20	14.5	219	16.9	235	28.2	475
	C58/J	a,c	14.8	65	16.3	215	9.9	96	13.2	102
	CBA/CaH	b	9.0	30	8.0	70	10.1	45	7.7	26
	C3H/HeJ	c	10.1	28	7.6	50	10.9	32	7.9	23
3	AKR/J	a	5.2	15	17.6	209	13.9	346	26.2	620
	CBA/J	a,c	6.3	25	20.9	291	11.5	170	25.8	542
	C58/J	a,c	8.2	98	18.6	171	13.3	116	17.2	208
	CBA/CaH	b	5.3	20	11.4	50	8.7	43	10.0	33
	C3H/HeJ	c	9.4	38	12.6	37	10.2	35	11.3	33

* B cells used as APC were prepared from spleen cells by treating spleen cells with rabbit anti-mouse brain antiserum plus complement, and were strained by DilC22(3). Mice used as a source of APC were injected with 200 μ l of goat anti-mouse IgD antiserum 24 h before mice were killed.

† Nylon wool-nonadherent, T cell-enriched, spleen cells were loaded with indo-1 AM.

§ V β 8.1 transgenic mouse (13).

|| Percent binding = Dil-positive T cells/total indo-1-positive T cells.

† Relative $[Ca^{2+}]_i$ increase = $[Ca^{2+}]_i$ of APC-bound T cells minus $[Ca^{2+}]_i$ of APC unbound T cells.

transgenic mice (Mls^b V β 8.1 TG) were used as a source of responder T cells. In this strain, >95% of splenic T cells express the V β 8.1 that is potentially Mls^a reactive (13). In multiple experiments, 20–40% of transgenic T cells are bound to Mls^a-expressing spleen cells and, in addition, a strong increase of $[Ca^{2+}]_i$ is observed in this population. In contrast to the high frequency of conjugates with Mls^a APC, T cell-APC conjugation and $[Ca^{2+}]_i$ elevation by T cells responding to the weak stimulatory Mls^c determinant are variable (Table 1 and Fig. 1). This difference may be explained solely by the presence of a much higher precursor frequency of Mls^a-reactive T cells than of Mls^c-reactive T cells. Alternatively, there may exist a unique property of Mls^a determinants that enhances T cell-APC conjugation, as originally proposed by Janeway et al. (25).

It was recently reported that Mls does not trigger the PI signaling pathway in T cell clones or hybridomas (26, 27). Since, in general, $[Ca^{2+}]_i$ elevation is tightly associated with PI hydrolysis in TCR-mediated signaling (28), PI hydrolysis was examined in unprimed heterogeneous T cells exposed to Mls stimulation. [³H]myo-inositol-labeled Mls^b V β 8.1 TG-T cells were mixed with B cells from Mls^{a+} CBA/J or

Mls^{a-} CBA/CaH, or with a polymeric form of anti-CD3 ϵ chain (2C11), and total PI hydrolysis was measured. Results of two independent experiments are shown in Table 2. Parallel to the T cell/APC conjugation and $[Ca^{2+}]_i$ elevation

Table 2. *PI Hydrolysis of V β 8.1 TG T Cells Stimulated with Mls^{a+} APC*

Stimulation*	Exp. 1	Exp. 2
None	528 [†]	309
2C11	3,955	4,782
2H6	416	258
CBA/J	1,428	4,425
CBA/CaH	861	479

* 7×10^6 T cells were stimulated with 50 μ g/ml heterodimeric anti-CD3 ϵ mAb (2C11), control mAb (2H6; hamster anti-arsenic acid), or 5×10^7 B cells.

† Total ³H-PI are mean for duplicate samples.

(Fig. 1), stimulation with Mls^{a+} CBA/J B cells but not with Mls^{a-} CBA/CaH B cells dramatically increased total water-soluble PI in Mls^b V β 8.1 TG T cells. These results are consistent with the conclusion that Mls stimulation triggers phospholipase C signal transduction pathway.

Differential Responses by Mature and Immature T Cells to Self-antigen. It has been shown that Mls antigens mediate the clonal deletion during intrathymic T cell development of cells

that express self-Mls-reactive TCR. We used this system to explore the interaction between T cells and their potential target cell in the process of T cell repertoire selection. As previously described, in $Mls^{a/b}$ V β 8.1 transgenic mice ($Mls^{a/b}$ V β 8.1 TG) generated by crossing the Mls^b V β 8.1 TG with Mls^{a+} CBA/J, the potentially self-Mls-reactive $CD4^+$ V β 8.1 $^+$ T cell subset is severely decreased in the periphery (13). In contrast to the extensive T cell-APC binding and

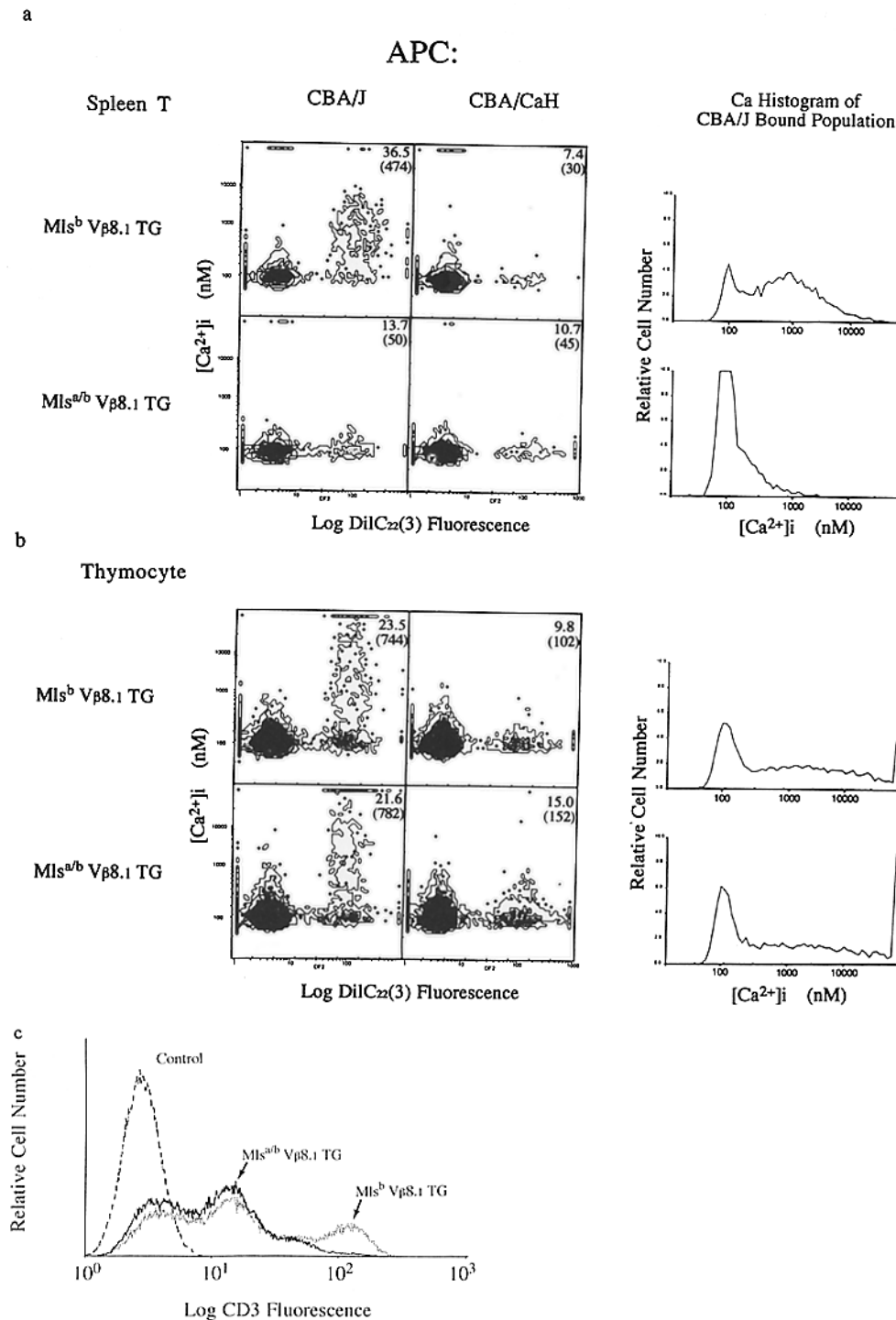


Figure 2. Immature thymocytes have reactivities to self-Mls determinants. (a) T cell/APC binding and [Ca²⁺]_i response of Mls^b or $Mls^{a/b}$ transgenic splenic T cells. 5×10^5 nylon wool-nonadherent T cells were mixed with 2×10^6 CBA/J (Mls^a) or CBA/CaH (Mls^b) APC. T cell/APC binding and [Ca²⁺]_i were examined by the procedure described in Materials and Methods. (Left) The x-axis indicates DilC22(3) fluorescence, which represents T cell-APC conjugates. The y-axis is the level of [Ca²⁺]_i of indo-1-loaded T cells. (Right) [Ca²⁺]_i distribution in response of bound population to CBA/J APC. The x-axis is the level of [Ca²⁺]_i (nM), and the y-axis indicates relative cell number. (b) T cell/APC binding and [Ca²⁺]_i response of Mls^b - or Mls^a -transgenic thymocytes. 5×10^5 thymocytes were mixed with 2×10^6 CBA/J (Mls^a) or CBA/CaH (Mls^b) APC. (c) Expression of CD3 by Mls^a or $Mls^{a/b}$ transgenic thymocytes. Thymocytes were stained with FITC-labeled hamster anti-mouse CD3 mAb, 2C11. A control with the FITC-labeled mouse anti-human CD3 mAb (....). $Mls^{a/b}$ transgenic thymocytes (—); Mls^b transgenic thymocytes (- - -).

strong $[Ca^{2+}]_i$ elevation (Fig. 2), and subsequent massive proliferative response of $Mls^b V\beta 8.1$ TG to Mls^{a+} APC (Fig. 3), splenic T cells from $Mls^{a/b} V\beta 8.1$ -TG generated no significant binding, $[Ca^{2+}]_i$ increase, or proliferative reactivity, indicating tolerance to self- Mls^a .

The staining of TG thymocytes with anti-CD3 antibody demonstrated that while $Mls^b V\beta 8.1$ TG thymus contains TCR bright-mature thymocytes and TCR dull-immature thymocytes, the high TCR mature population was selectively reduced in the $Mls^{a/b} V\beta 8.1$ (Fig. 2 c). As observed in splenic T cells, thymocytes from Mls^b TG showed a strong proliferative response to Mls^{a+} APC, whereas no significant proliferation of $Mls^{a/b}$ thymocytes is observed. The similar proliferative responses of thymocyte from these two transgenic mice to a mixture of ionomycin and PMA indicated that the proliferative ability of these two thymocyte populations did not differ. However, the pattern of binding and $[Ca^{2+}]_i$ response of TG thymocytes to self- Mls^a antigen is dramatically different. As shown in Fig. 2 b, not only Mls^b TG thymocytes but also $Mls^{a/b}$ TG thymocytes bound Mls^{a+} APC and exhibited a $[Ca^{2+}]_i$ elevation. These results suggested that immature T cells bearing potentially self-reactive TCR are able to recognize self-antigen and trigger signal transduction, but this signaling does not result in cellular proliferation.

To further characterize the mechanism underlying the response (and potentially the clonal deletion) of self-reactive

T cells, the roles of several cell surface molecules were examined (Fig. 4). The binding of Mls^{a+} APC and the subsequent $[Ca^{2+}]_i$ increase in $Mls^b V\beta 8.1$ TG splenic T cells are strongly inhibited by anti-CD4 but minimally by anti-CD8, indicating a preferential role of CD4 molecules (24) in T cell Mls^a recognition. Furthermore, splenic T cells are inhibited by mAb F23.1, reactive to the TG TCR, $V\beta 8.1$, but not by F23.2, specific for $V\beta 8.2$. Antibodies that recognize class II antigens, I-A^k and I-E^k, expressed by Mls^{a+} APC, and antibodies reactive to integrins, LFA-1 and ICAM-1, are also strongly inhibitory, suggesting that antigen-specific TCR/ligand interaction and adhesion molecules are crucial for the recognition of Mls^a . Antibodies specific for TCR, class II, and integrins also inhibit self- Mls recognition by thymocytes of $Mls^{a/b} V\beta 8.1$ TG as measured by T cell-APC conjugation, suggesting that these molecules are similarly essential for thymocyte recognition and selection. Interestingly, the APC binding and $[Ca^{2+}]_i$ elevation of $V\beta 8.1$ TG thymocytes, which include TCR bright cells, and peripheral T cells was blocked by anti-CD4 mAb, but the APC binding of $Mls^{a/b} V\beta 8.1$ TG thymocytes, which lack TCR bright cells, required the combination of anti-CD4 and anti-CD8 mAbs for maximal inhibition. In contrast, anti-CD4 alone did strongly inhibit the $Mls^{a/b} V\beta 8.1$ TG thymocyte $[Ca^{2+}]_i$ response (Fig. 4). These results suggested the important role of both CD4 and CD8 molecules for clonal deletion of self- Mls -reactive T cells.

Discussion

Using a newly established cellular binding assay system, we have studied the induction of TCR-mediated signaling in mature as well as immature T cells by endogenous superantigen, the Mls gene product.

The early signal transduction events associated with T cell recognition of superantigens are not well understood. For example, whether T cells utilize an identical signaling pathway in response to these superantigens to that in response to conventional peptide antigens is a matter of controversy. It was recently reported that Mls does not induce increased $[Ca^{2+}]_i$ (29) or PI hydrolysis (26, 27) in T cell clones or hybridomas, and it was proposed on this basis that superantigen stimulation does not trigger the phospholipase C signal transduction pathway. In contrast, we constantly observed strong Ca response in Mls -specific response of heterogeneous (Fig. 1) as well as Mls -specific T cell clones (Ishida et al., manuscript submitted for publication). We also observed a specific increase in PI hydrolysis after interaction of heterogeneous T cells from $Mls^b V\beta 8.1$ TG (Table 2) and cloned T cells (Ishida et al., manuscript submitted for publication) with Mls^{a+} stimulators. This apparent discrepancy between our results and previous reports may be explained by differences in the Mls^{a+} APC used. To maximize Mls -specific stimulation, we used activated B cells as a source of Mls^{a+} -APC by injecting antibody specific for mouse IgD in vivo, a procedure that enhances the capacity of splenocytes to stimulate across an Mls difference without altering the specificity of

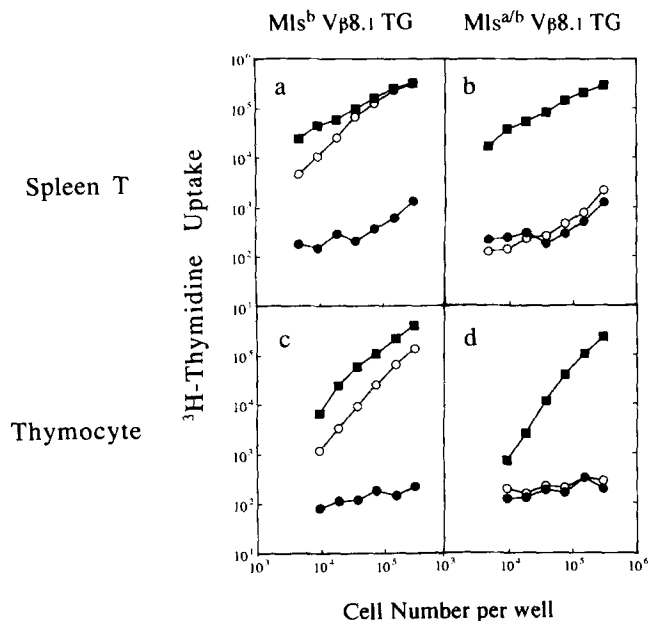


Figure 3. Proliferative response of splenic T cells and thymocytes from Mls^a or $Mls^b V\beta$ TG to Mls^{a+} APC. Varying number of splenic T cells (a and b) or thymocytes (c and d) were cultured with 5×10^5 B cells inactivated by 3,000-rad irradiation from CBA/J (O) or CBA/CaH (●). As a control, phorbol esters (PMA) and calcium ionophores were added at the final concentration of 10 and 20 ng/ml, respectively (□). Each point represents the mean of $[^3H]$ TdR incorporation of triplicate cultures. T cell proliferation was assayed as described previously (24).

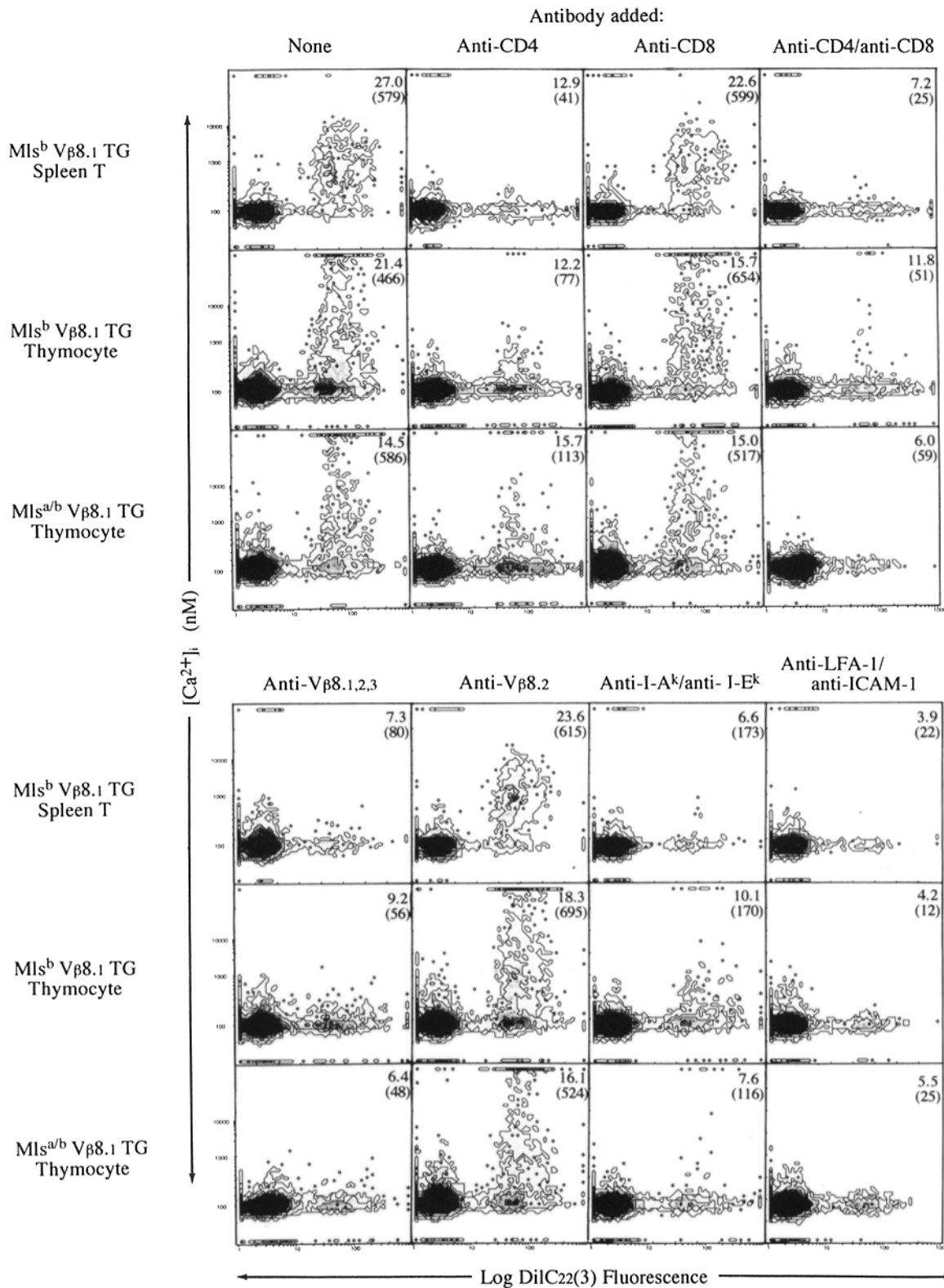


Figure 4. The role of cell surface molecules in T cell/APC binding and $[Ca^{2+}]_i$ response. Splenic T cells or thymocytes from Mls^b or Mls^{a/b} Vβ8.1 TG were mixed with CBA/J APC in the presence of designated antibodies. Rat anti-CD4 mAb, GK1.5, 40 μg/ml, anti-CD8:1/50 ascites of mouse anti-Lyt2.1 mAb; anti-Vβ8.1,2,3 mAb, 40 μg/ml of protein A-purified F23.1; anti-Vβ8.2 mAb, 40 μg/ml of protein A-purified F23.2; anti-I-A^k/Anti-I-E^k mAb, 10 μg/ml of protein A-purified 10-2-16 and 14-4-4; anti-LFA-1/anti-ICAM-1 mAb, 20 μg/ml of protein G-purified FD441.8 and YN1/1.7.4.

the response (21). It is conceivable that the interaction of Mls with the TCR is lower than that of other antigens, so that activation of the APC in vivo or in vitro increases the avidity of the Mls/TCR interaction by altering expression of cell adhesion molecules or by influencing expression of MMTV provirus product. Consistent with this hypothesis, we also observed only low levels of T cell-APC conjugation and $[Ca^{2+}]_i$ increase of Mls^a-reactive T cells with Mls^a-APC obtained from a normal mouse. Overall, our results indicate that Mls determinants trigger the conventional phospholipase C pathway for T cell signal transduction.

Consistent with the strong primary MLR, heterogeneous T cells from normal mice reveal massive Mls^a-specific T cell/APC conjugation and $[Ca^{2+}]_i$ response (Table 1 and Fig. 1). In contrast, those populations do not exhibit constant T cell/APC conjugation and $[Ca^{2+}]_i$ elevation in response to Mls^c or allo-MHC gene products (data not shown). A higher precursor frequency of Mls^a-reactive T cells than Mls^c- or allo-MHC-reactive T cells in normal mice apparently contributes to this different reactivity. In addition, qualitative and quantitative difference between those allo-antigens may be also involved. Among Mls^c-specific T cell responses, C58/J constantly elicits significant T cell/APC conjugation as well as $[Ca^{2+}]_i$ elevation, whereas, despite the expression of Mls^c, CBA/J and C3H/HeJ show weak or no effect to AKR/J T cells. Loci encoding Mls^c determinants in these three strains are mapped to different mouse mammary tumor virus (MMTV) provirus, C58/J to MTV-3 (30), CBA/J to MTV-6, and C3H/HeJ to Mtv-1 and Mtv-6 (31). Mtv-3 may somehow interact with the Mls^c-reactive V β 3 segment more efficiently than other MMTV gene products.

It was noticed that despite the fact that the majority of splenic T cells from transgenic mice express potentially Mls^a-reactive V β 8.1, only 20–40% of T cells reveal specific binding to Mls^a APC (Table 1). This lower than expected ratio of Mls^a-binding cells is partially explained by the preferential APC binding of CD4⁺ T cell (32). Enrichment of CD4⁺ population by cytolytic treatment of splenic T cells with anti-CD8 antibody increased the binding as well as $[Ca^{2+}]_i$ response, whereas the CD4-depleted population revealed a much weaker response to Mls^a APC (data not shown), suggesting that in an initial Mls^a-reactive T cell response, only a small proportion of CD8⁺ T cells have the ability to induce $[Ca^{2+}]_i$ dependent early signal transductions. The almost complete inhibition of the response of unfractionated Mls^b V β 8.1 T cells with anti-CD4 antibody but not anti-CD8 antibody is consistent with a preferential role of CD4 molecules in this early event of T cell Mls^a recognition. However, it should be noted that significant populations of CD4⁺ T cells neither form conjugates nor elicit $[Ca^{2+}]_i$ ele-

vation against Mls^a APC. The nature of these functionally distinct CD4⁺ V β 8.1-bearing T cells is currently being investigated.

Peripheral T cells from Mls^{a/b} V β 8.1 TG, which express the potentially self-reactive TCR, show no reactivities to Mls^a. In contrast, thymocytes from these animals do not proliferate to self-Mls antigens, but a proportion of immature thymocytes do form specific conjugates with self-Mls^a APC and show a strong $[Ca^{2+}]_i$ response. These results suggest that during the course of negative selection, immature T cells that bear potentially self-reactive TCR specifically interact with self-ligand and enter an initial activation stage, but do not proliferate and expand. Instead, this interaction may lead to the delivery of signals to undergo programmed cell death, which play a role in negative selection (33, 34). It should be pointed out that the $[Ca^{2+}]_i$ distribution of Mls^b V β 8.1 TG peripheral T cells bound to APC is more homogeneous than that of APC binding thymocytes from mice of either Mls type. This suggests an intrinsic difference in $[Ca^{2+}]_i$ regulation during activation between peripheral and intrathymic T cell populations and could be related to the change in response to $[Ca^{2+}]_i$ elevation during T cell differentiation.

The self-reactivity of thymocytes is inhibited by antibodies specific for TCR, CD4, CD8, class II molecules, LFA-1, and ICAM-1, suggesting a critical role of these molecules for tolerance induction. In particular, both CD4 and CD8 appear to function in the interaction of immature thymocytes with a class II-restricted self-ligand, suggesting that both CD4 and CD8 support the intimate interaction of immature CD4⁺ CD8⁺ T cells with a ligand in the thymic environment through the TCR. If a ligand is efficiently presented in association with MHC products, e.g., with class II for Mls determinant, a tolerogenic signal that leads potentially autoreactive T cells to programmed cell death (4, 33, 34) would be provided by this CD4/TCR-ligand interaction. This may be the mechanism by which T cell bearing potentially autoreactive TCR are eliminated at the stage of CD4⁺ CD8⁺ thymocyte development, thereby resulting in the deletion of self-Mls-reactive V β s in both CD4⁺- and CD8⁺-peripheral T cells (33).

Using a newly established cellular binding assay system, we have demonstrated that TCR, CD4 accessory molecules, and LFA-1 play an equally essential role for T cell antigen recognition and activation in the periphery, and we speculate that these molecules may play a similar role during induction of self-tolerance by interacting with their ligand molecules. We believe that this system can be applied to a wide variety of studies involving receptor/ligand interaction under physiological conditions.

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