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Unique molecular surface features of *in vivo* tolerized T cells

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ABSTRACT **Differential expression of surface markers can frequently be used to distinguish functional subsets of T cells, yet a surface phenotype unique to T cells induced into an anergic state has not been described. Here, we report that CD4 T cells rendered anergic *in vivo* by superantigen can be identified by loss of the 6C10 T cell marker. Inoculation of Vβ8.1 T cell antigen receptor (TCR) transgenic mice with a Vβ8.1-reactive minor lymphocyte-stimulating superantigen (Mls-1^a) induces tolerance to Mls-1^a by clonal anergy. CD4 lymph node T cells from Mls-1^a inoculated transgenic mice enriched for the 6C10$^-$ phenotype neither proliferate nor produce interleukin-2 upon TCR engagement, whereas 6C10$^+$ CD4 T cells retain responsiveness. Analysis of T cell memory markers demonstrate that 6C10$^-$ T cells remain 3G11^{hi} but express heterogeneous levels of CD45RB, CD62L, CD44, and the CD69 early activation marker, suggesting that T cells at various degrees of activation can be functionally anergic. These studies demonstrate that anergic T cells can be purified based on 6C10 expression permitting examination of issues concerning biochemical and biological features specific to T cell anergy.**

T cells can be induced into an anergic state in which they do not proliferate to subsequent antigen stimulation (1). Induction of T cell anergy may be an important process in the prevention of autoimmune responses against peripheral self-antigens by T cells that are not centrally tolerized. Bacteria and viruses express superantigens that can induce clonal anergy and render the host tolerant to these antigens (for review see ref. 2). Superantigens bind directly to select β-variable (V) chains of T cell antigen receptors (TCR) independent of the peptide antigen binding site and either delete or anergize the reactive T cells. The retrovirally encoded minor lymphocyte stimulating superantigen (Mls-1^a) engages T cells that express Vβ6 and 8.1 TCR chains (3, 4). We have previously reported that a majority of peripheral CD4 T cells from a Vβ8.1 transgenic mouse are rendered anergic by exogenous Mls-1^a (5).

Progression of T cell functional responses during the induction of tolerance to Mls-1^a is reasonably well characterized and similar in both TCR transgenic and nontransgenic mice (5–9). Inoculation of Vβ8.1 transgenic mice on the CBA/CaH background (H-2^k, Mls-1^a negative) with T cell-depleted splenocytes from CBA/J mice (H-2^k, Mls-1^a positive) initially invokes a strong proliferative T cell response and expansion of the Vβ8.1$^+$ T cells during the first 3 days (5). This is followed by a deletion phase (10, 11), during which the number of Vβ8.1$^+$ T cells returns to normal levels. Tolerance is achieved during this period, and by day 14 postinoculation, a majority of the surviving CD4 T cells are anergic. Most peripheral T cells remain refractory to *in vitro* stimulation for at least 30 days after administering Mls-1^a.

Anergic T cells can be identified functionally because, unlike memory cells, they neither proliferate nor produce interleukin (IL)-2 upon subsequent antigen challenge. However a surface phenotype that can distinguish anergic cells from non-anergic T cells has not been described, making it difficult to separate T cells induced into an anergic state from those that have escaped anergy induction.

The 6C10 marker is a phosphaditylinositol-anchored glycosylated molecule whose expression is associated with Thy-1 expression and may represent a differentially glycosylated form of Thy-1 that is not expressed on all T cells (12). Previously, we reported that very small numbers of nonproliferative CD4 T cells can be isolated from normal, unperturbed mice by sorting for peripheral T cells that have lost the expression of both 6C10 and 3G11 (13–15). T cells that are 6C10$^-$3G11^{lo} seem to have undergone prior activation in that they express low levels of CD45RB and CD62L and high levels of CD44 (14, 16, 17). It is possible then that 6C10$^-$3G11^{lo} T cells have arisen from the naive 3G11^{hi} population and have been induced into an anergic state by peripheral mechanisms. Here, we examine the expression of 6C10 and 3G11 on peripheral CD4 T cells rendered anergic by introducing Mls-1^a into Vβ8.1 transgenic mice.

MATERIALS AND METHODS

Mice. CBA/CaH (H-2^k, Mls-1^a negative) and CBA/J (H-2^k, Mls-1^a) mice were obtained from the National Cancer Institute (NIH, Bethesda, MD). Vβ8.1 transgenic mice were bred onto a CBA/CaH background and express the transgene on greater than 95% of peripheral T cells (8).

Tolerance Induction and CD4 T Cell Recovery. Tolerance to Mls-1a was induced as previously described (5) by inoculating Vβ8.1 transgenic mice with a single intravenous injection of 1.5×10^7 CBA/J splenocytes depleted of T cells. The inguinal, popliteal, brachial, axillary, and cervical lymph nodes were harvested, and the CD4 T cells were purified by depleting non-CD4 cells with complement fixation and antibodies directed against heat-stable antigen (J11d), I-E^k (14-4-4), and CD8 (3.155) and centrifuging over a cushion of lympholyte M (Cedarlane Laboratories). On average, this CD4 T cell preparation from uninoculated transgenic mice yielded 97% CD4$^+$ purification of the viable cells, whereas 90% of the viable cells from tolerant mice were CD4$^+$. The purified T cells were

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Abbreviations: TCR, T cell antigen receptor; Mls, minor lymphocyte-stimulating antigen; IL, interleukin; FITC, fluorescein isothiocyanate.

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separated into 6C10$^-$ and 6C10$^+$ populations by MACS magnetic cell sorting, using a Type AS column and rat-anti-mouse-IgM MicroBeads following the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). On average, magnetic sorting for 6C10 expression yielded populations that were 80% 6C10$^-$ and 93% 6C10$^+$, respectively.

Flow Cytometry. 5×10^5 purified CD4 T cells were stained with three or four colors: anti-CD4 PE (GIBCO-BRL), biotin anti-Vβ8.1/2 (clone MR5–2, PharMingen) and streptavidin-RED670 (GIBCO-BRL), and 6C10 or 3G11 supernatant and anti-IgM fluorescein isothiocyanate (FITC) (Southern Biotechnology Associates). Biotinylated anti-Vβ8.1 was substituted with anti-Vβ6 biotin (clone RR4–7, PharMingen), anti-Vβ14 biotin (clone 14–2, PharMingen), CD62L biotin (L-selectin, clone MEL-14, PharMingen), CD44 biotin (Pgp-1, clone IM7, PharMingen), CD69 biotin (clone H1.2F3, PharMingen), or CD25 biotin (IL-2 receptor α-chain, p55, clone 7D4, PharMingen) to assess the expression levels of other T cell markers. CTLA-4 (clone UC10–4F10, a generous gift from L. Turka, University of Pennsylvania) was detected with anti-hamster IgG FITC (Southern Biotechnology Associates), and CD45RB (clone 23G2, PharMingen) was directly coupled to FITC; these two antibodies were used in combination with 6C10/anti-IgM biotin (Southern Biotechnology Associates) or 3G11 biotin (PharMingen).

Purified CD4 T cells from tolerant Vβ8.1 transgenic mice that received Mls-1^a 14–30 days earlier were sorted into 6C10$^-$ and 6C10$^+$ populations and stimulated *in vitro* with 1 μg/ml 145–2C11 and T-depleted, irradiated syngeneic splenocytes. CD69 expression was analyzed after 12–24 hr of *in vitro* stimulation, and CTLA-4 and CD25 expression was analyzed after 40 hr.

For all flow cytometry, 1 nM TO-PRO-3 (Molecular Probes) was added to each tube just before analysis; TO-PRO-3 is excluded by viable cells and is activated by the red diode laser (635 nm) of FACSCalibur (Becton Dickinson). Cells positive for CD4 and negative for TO-PRO-3 were gated for analysis of expression of other markers. Flow cytometry data were acquired at the Flow Cytometry and Cell Sorter Core Facility (Cancer Center, University of Pennsylvania) and analyzed using CELLQuest.

Proliferation Assays. Purified CD4 T cells were plated (5×10^4/well) in triplicate with 1) T cell-depleted, irradiated (2000 R) CBA/J stimulators (5×10^5, unless otherwise indicated) for 72 hr, 2) syngeneic splenocytes (5×10^5) plus soluble anti-CD3 (145–2C11) for 48 hr, or 3) 100 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 200 nM calcium ionophore (ionomycin, Sigma) for 36 hr total, with a pulse of 1 μCi/well [^{3}H]TdR during the last 8–12 hr. Each assay was repeated four times with similar results. Supernatants from anti-CD3-stimulated T cells were harvested at 48 hr, and the production of IL-2 was determined by measuring proliferation of the IL-2-dependent cell line CTLL-2 in the presence of anti-IL-4 antibody (11B11), where 1 unit is the amount of IL-2 required to support half-maximal [^{3}H]TdR incorporation (18).

RESULTS

We found that changes in 6C10, but not 3G11, expression accompanies induction of tolerance to Mls-1^a in Vβ8.1 transgenic mice (Figs. 1 and 2). During the first 3 days after Mls-1^a inoculation there is a progressive reduction in the percent of Vβ8.1$^+$ CD4 T cells that express 6C10 as well as a reduction in the percent of 3G11^{hi}. However, the percent of T cells expressing 3G11 returns to normal levels during the next 10 days, long before tolerance to Mls-1^a is broken (Fig. 2). The low percentage of Vβ8.1$^+$ CD4 T cells expressing 6C10, on the other hand, is maintained for at least 30 days, as is tolerance to Mls-1^a. By 120 days postinoculation, 6C10 expression recovers on 74% of the CD4 lymph node T cells, and tolerance to Mls-1^a is reversed (data not presented in figures).

We examined alterations of 6C10 and 3G11 expression during the induction of tolerance to Mls-1^a in nontransgenic CBA/CaH mice to determine whether the TCR transgene was causing aberrant T cell responses. T cells that responded to the superantigen (Vβ6$^+$ T cells) expanded as expected (7) and changes in 6C10 and 3G11 expression paralleled changes in the Vβ8.1 transgenic mice, whereas negligible modifications were found on T cells that do not respond to Mls-1^a (Vβ14$^+$ T cells)

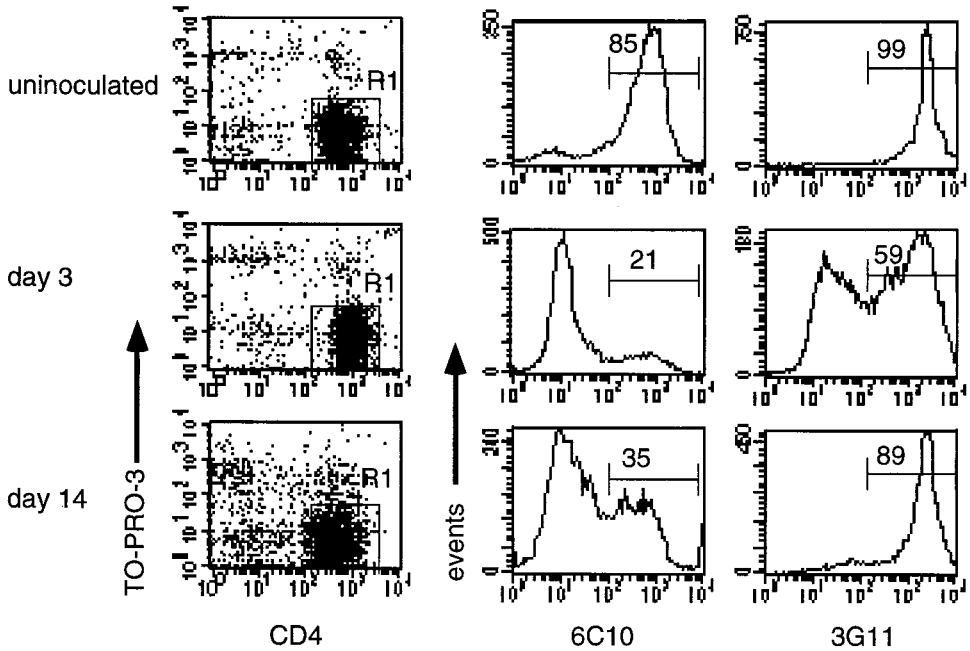

FIG. 1. Analysis of changes in 6C10 and 3G11 expression on tolerant CD4 lymph node T cells. Vβ8.1 transgenic mice were tolerized by inoculating with Mls-1^a prior to days indicated, and expression of 6C10 and 3G11 on purified CD4 lymph node T cells was determined by flow cytometry, gating on CD4$^+$ and TO-PRO-3$^-$ cells. The percent of 6C10$^+$ and 3G11^{hi} CD4 T cells is indicated by the number above the brackets. Representative flow cytometry profiles are shown.

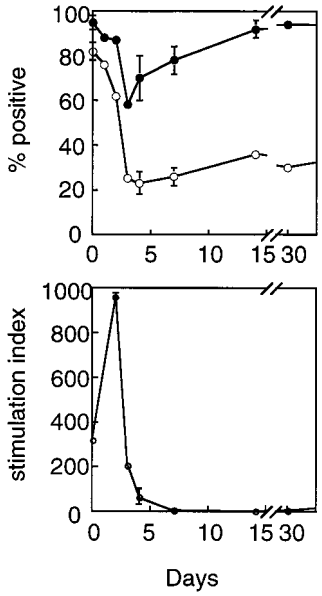

FIG. 2. 6C10, but not 3G11, down-modulation on CD4 T cells is correlated with duration of tolerance to Mls-1^a. (*Upper*) Percent of 6C10$^+$ (○) and 3G11^{hi} (●) CD4 T cells as determined by flow cytometry, on the days indicated after Mls-1^a inoculation (day 120 data not shown). (*Lower*) Proliferation of purified CD4 T cells in response to Mls-1^a *in vitro*. Results are expressed as stimulation indices of responses to CBA/J stimulators (day 0 T cells, which incorporated approximately 80,000 cpm) divided by responses to syngeneic CBA/Ca stimulators and represent averages ± SEM of four experiments.

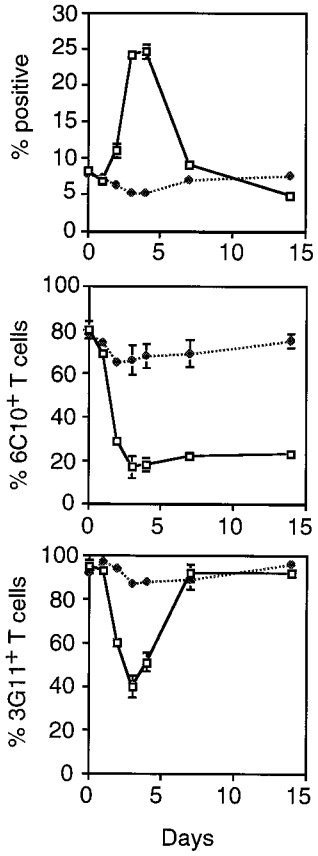

FIG. 3. Modulation of 6C10 and 3G11 expression on Mls-1^a reactive CD4 lymph node T cells from nontransgenic mice after administering Mls-1^a parallels changes in 6C10 and 3G11 expression in Vβ8.1 transgenic mice. Nontransgenic CBA/CaH mice were inoculated with Mls-1^a prior to the day indicated and percent of Vβ6$^+$ (□) and Vβ14$^+$ (◆) T cells (*Top*); Vβ6$^+$ and Vβ14$^+$ T cells expressing 6C10 (*Middle*) and 3G11 (*Bottom*) determined by flow cytometry.

(Fig. 3). Therefore, alteration of the expression of 6C10 and 3G11 is specific for CD4 T cells responding to the tolerizing antigen and is not a property associated uniquely with this particular TCR transgene. The correlation between the loss and reacquisition of 6C10 expression with the duration of tolerance to Mls-1^a suggests that anergic cells can be identified through down-modulation of 6C10.

T cells that no longer expressed 6C10 in Mls-1^a tolerant Vβ8.1 transgenic mice were sorted from 6C10$^+$ T cells (Fig. 4*A*) to examine the functional state of 6C10$^-$ T cells. 6C10$^-$ cells still expressed high levels of surface TCR (Fig. 4*A*) but proliferated negligibly to anti-CD3 stimulation (Fig. 4*B*) and, by the CTLL-2 assay, produced undetectable levels of IL-2, indicating that the cells were anergic. The 6C10$^+$ population, on the other hand, proliferated to anti-CD3 stimulation in a dose-dependent manner similar to the control CD4 T cells from uninoculated mice (Fig. 4*B*) and produced half the level of IL-2 (13 and 24 U/ml, respectively). Furthermore, 6C10$^+$ T cells proliferated strongly when stimulated with Mls-1^a, whereas proliferation in the 6C10$^-$ T cells was negligible (Fig. 4*C*). There was no significant difference between the proliferation of 6C10$^-$, 6C10$^+$, and control populations in response to phorbol 12-myristate 13-acetate and calcium ionophore (ionomycin) (Fig. 4*D*), indicating that 6C10$^-$ cells can respond to mitogenic stimuli that bypass the TCR. These data demonstrate that CD4 lymph node T cells induced into a state of anergy by Mls-1^a can be recognized and purified by the loss of 6C10 expression.

Analysis of T cell surface markers on the 6C10$^-$ T cells from tolerant mice show that a portion of anergic T cells express low levels of CD45RB and CD62L and high levels of CD44, indicating prior activation or memory (Table 1), even though a recall response to Mls-1^a is not found *in vitro* (Fig. 1 *Lower*) or *in vivo* (19, 20). Furthermore, several 6C10$^-$ T cells have up-regulated the transiently expressed CD69 T cell activation marker (21) *in vivo* compared with 6C10$^+$ cells (Table 2). Yet, many 6C10$^-$ T cells also retain a naive phenotype, suggesting that anergic T cells can be found in various states of activation.

Anergic 6C10$^-$ T cells from tolerant mice do not proliferate in response to *in vitro* anti-CD3 stimulation but do undergo some responses to TCR ligation. Surface expression of CD69 and CTLA-4 (22) are up-regulated, whereas the expression of the high affinity p55 IL-2 receptor α chain CD25 is minimally changed (5, 23) on the 6C10$^-$ cells as a consequence of *in vitro* anti-CD3 stimulation (Table 2). These data suggest that anergic T cells can respond to environmental cues as would normal T cells, but the TCR has been uncoupled from signaling machinery necessary for proliferation.

DISCUSSION

Here, we report that expression of the 6C10 T cell marker is down-regulated on T cells that have been induced into an anergic state by Mls-1^a. We previously reported that T cells isolated from unperturbed BALB/c mice that were negative for both 6C10 and 3G11 were refractory to further stimulation, suggesting that they might represent T cells induced into an anergic state in normal situations. The premature recovery of 3G11 on peripheral cells from Mls-1^a inoculated mice suggests that 3G11 expression is not associated with T cell anergy but instead might reflect other features of T cell stimulation. T cells that have down-modulated 3G11 expression in response to Mls-1^a inoculation disappear during the deletion phase, suggesting that these are the cells undergoing activation-induced apoptosis (10, 11) or no longer migrate to the lymph

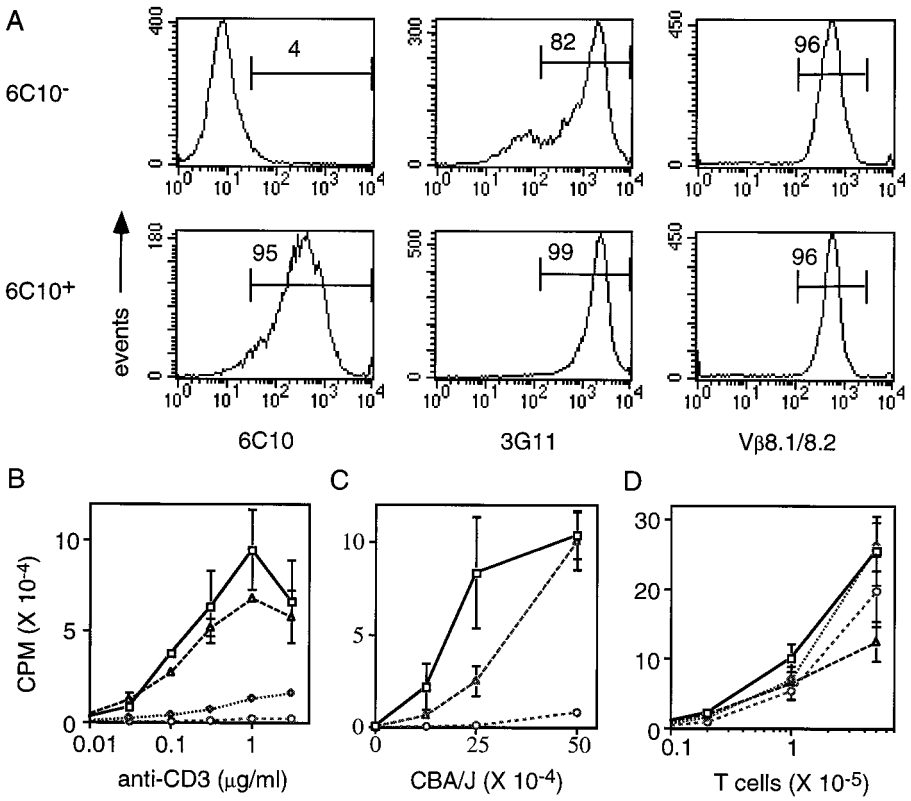

FIG. 4. 6C10$^-$ CD4 lymph node T cells from Mls-1^a inoculated Vβ8.1 transgenic mice are found to be functionally anergic. CD4 T cells from tolerant mice were purified into 6C10 positive and negative populations by magnetic sorting and analyzed for 6C10, 3G11, and Vβ8.1 expression by flow cytometry (*A*), dose-dependent proliferation to anti-CD3 stimulation [uninoculated mice (□) and inoculated mice unsorted (◇), purified 6C10$^-$ (○), and 6C10$^+$ (△) (*B*), dose-dependent proliferation to CBA/J stimulators (legend as for *B* except inoculated unsorted is not shown) (*C*), and proliferative response to phorbol 12-myristate 13-acetate/ionomycin (*D*). Representative data from experiments repeated four times are shown.

nodes (2). However sorting 6C10$^-$ T cells from those retaining 6C10 expression in Mls-1^a inoculated Vβ8.1 transgenic mice clearly segregates peripheral CD4 T cells into distinct anergic and non-anergic populations.

The entire 6C10$^-$ peripheral T cell population from uninoculated Vβ8.1 transgenic mice are not anergic and can proliferate and produce IL-2 in response to *in vitro* stimulation nearly as well as 6C10$^+$ T cells (data not shown and ref. 13). T cell depletion studies *in vivo* suggest that recent thymic emigrants are 6C10$^-$3G11^{hi}, and 6C10 expression becomes apparent on peripheral T cells in an antigen-independent fashion, indicating that naive cells can be both negative or positive for 6C10 expression (13–15). We found that inoculation of Mls-1^a into thymectomized Vβ8.1 transgenic mice induced down-regulation of 6C10 expression to similar extents as non-thymectomized mice (data not shown), indicating that the 6C10$^-$3G11^{hi} population in tolerant mice does not represent recent thymic emigrants. Therefore, anergic 6C10$^-$ T cells from tolerant Vβ8.1 transgenic mice represent a population of T cells functionally distinct from naive 6C10$^-$ T cells of normal mice.

Heterogeneous expression of memory markers on 6C10$^-$ T cells from Mls-1^a tolerant mice suggests that either maturation of anergic T cells is arrested at various stages after Mls-1^a stimulation or that the 6C10$^-$ T cells represent a dynamic population maintained in a nonproliferative state by constant antigen stimulation. Others have found that the surface phenotype of Vβ6$^+$ T cells from Mls-1^a tolerant mice that had previously proliferated as determined by BrdU incorporation *in vivo* also resembled both naive and memory T cells (20). These data support the idea that Mls-1^a induces various degrees of maturation even in T cells expressing an Mls-1^a reactive transgenic TCR Vβ chain. Responses include deletion of cells that down-modulate 3G11, anergy in cells losing 6C10, and limited activation of cells retaining 6C10. In addition, we consistently see at least a 2-fold increase in the number of cells that are negative for CD4 expression in mice that have been dosed with Mls-1^a (Fig. 1). These cells express the Vβ8.1 transgene but do not express CD8 (data not shown) and are therefore double-negative peripheral T cells. It is possible that these cells are similar to the double-negative T cells from SEA-treated TCR transgenic mice that were demonstrated by Swain's group to produce high levels of interferon-γ and suppress responses of the CD4$^+$ T cells from these mice (24). We have not yet examined these cells in detail; however, we did not find a correlation between 6C10 expression and the T cells that have the double-negative phenotype in our tolerance system. The different fates of T cells from Mls-1^a inoculated Vβ8.1 transgenic mice might reflect affinity differences of the TCR caused by distinct Vα chain usage (8, 25–28).

We have found that 6C10$^+$ T cells recovered from tolerant mice can respond to Mls-1^a; therefore, the Vα chain has not completely disrupted reactivity with Mls-1^a, at least *in vitro*. The 6C10$^+$ T cells might have escaped anergy induction for several possible reasons, none of which are mutually exclusive, all of which assume limited exposure to the superantigen. *In*

Table 1. Anergic CD4 lymph node T cells (6C10$^-$) express markers indicating both memory and naive T cell phenotype

	CD62L^{lo}	CD44^{hi}	CD45RB^{lo}
Uninoculated	9 ± 2	10 ± 1	9 ± 0
Inoculated, 6C10$^-$	34 ± 4	29 ± 1	19 ± 2
Inoculated, 6C10$^+$	22 ± 1	12 ± 4	13 ± 5

Values represent percent of cells expressing markers at levels that suggest prior activation as determined by flow cytometry and are averages ± SEM of three experiments.

Table 2. Anergic CD4 lymph node T cells ($6C10^-$) up-regulate T cell activation markers following *in vitro* anti-CD3 stimulation

	$CD69^+$		$CD25^+$		$CTLA\text{-}4^+$	
anti-CD3	−	+	−	+	−	+
Uninoculated	8 ± 1	78 ± 13	4 ± 2	77 ± 13	2 ± 1	94 ± 2
Inoculated, $6C10^-$	20 ± 1	59 ± 15	3 ± 1	37 ± 14	6 ± 4	90 ± 4
Inoculated, $6C10^+$	13 ± 0	74 ± 16	10 ± 1	73 ± 17	2	92 ± 2

Values represent percent of cells positive for CD69, CD25, or CTLA-4 as determined by flow cytometry and are averages ± SEM of three experiments.

vitro, the $6C10^+$ T cells require twice the antigen to achieve responses similar to the control T cells, suggesting that the TCR has somewhat reduced affinity for Mls-1^a, or the $6C10^+$ T cells might have arisen after the bulk of the Mls-1^a antigen had cleared from the inoculated animals, or perhaps these T cells were sequestered from the antigen.

The regulatory role of anergic cells, if any, remains unclear; nevertheless, their ability to modulate surface receptors involved in homing and other regulatory functions as well as their ability to synthesize cytokines, such as interferon-γ (data not shown and refs. 15, 29–31) suggest that anergic cells are not passive bystanders but instead can play an active role in modulating immune responses, possibly by mediating suppressive activities in the periphery (32). The ability to purify anergic T cells from *in vivo* sources based on loss of 6C10 expression will enable studies on these biological issues as well as biochemical studies on altered TCR-mediated mechanisms.

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