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# Following Antigen Challenge, T Cells Up-Regulate Cell Surface Expression of CD4 In Vitro and In Vivo<sup>1</sup>

William Ridgway, Marcella Fassò, and C. Garrison Fathman<sup>2</sup>

The low precursor frequency of Ag-specific T cells has raised significant barriers to studying the T cell response in vivo. We demonstrate that T cells up-regulate the cell surface expression of CD4 following Ag recognition, which identifies Ag-specific T cells in vitro and in vivo and allows their characterization. The CD4<sup>high</sup> cell subpopulation contains the Ag-specific population as indicated by Ag-induced proliferation and limiting dilution analyses. The use of the CD4<sup>high</sup> marker will allow analysis of the dynamics of the T cell immune response in vivo, the study of the suboptimal T cell response to Ag, and the identification of T cells which are reactive to known and unknown autoantigens. *The Journal of Immunology*, 1998, 161: 714–720.

The immune response to foreign protein Ags is dependent upon Ag-specific CD4<sup>+</sup> T cells. However, the frequency of T cells that are specific for any particular Ag is extremely low (on the order of 1:50,000–300,000 T cells in unprimed individuals and ~1:1000–10,000 T cells in primed individuals) (1–4), which makes their analysis extremely difficult. Although several methods have been developed to study T cell responses in vivo (e.g. the adoptive transfer of T cell clones and TCR transgenic T cells) (5–7), and some T cell responses to particular peptide Ags (e.g. peptides from pigeon cytochrome *c*) use highly restricted TCRs which enable the tracking of Ag responses with clonotypic Abs (8–12), the characterization of Ag-specific T cell responses requires a manipulation of the normal physiology of the immune response. Thus, although these methods have provided important insights into T cell responses in vivo, they share some drawbacks in studies of the conventional immune response.

T cell clones and T cells from TCR transgenic mice use a single TCR  $\alpha\beta$  heterodimer; these T cells generate a homogenous, single affinity response to Ag. The methods used to isolate both T cell clones and the T cells from which TCR transgenic mice are derived have used culture conditions that are predisposed to selecting only the best growing T cells from culture (i.e., the T cells with the proliferative response that is “most favorable” for growth). mAbs to clonotypic TCR  $\alpha$ - and  $\beta$ -chains can be used to study T cell immune responses in vivo, but only to those Ags that are known to elicit a clonotypic response can be studied; moreover, the Abs will only identify T cells with the clonotype discovered in vitro, thereby confounding the question of whether the T cell response in vivo is similar to the T cell response in vitro.

We have identified a novel way to investigate the response of Ag-specific T cells in vitro and in vivo that avoids these drawbacks. Here, we demonstrate a model system, using the DBA/2

response to sperm whale myoglobin (SWM)<sup>3</sup>(110–121), to study Ag-specific T cells in vitro and in vivo. Our data demonstrate that Ag-specific murine T cells up-regulate their surface expression of the CD4 molecule following Ag recognition in vitro and in vivo. The CD4<sup>high</sup> cells contain all of the Ag-specific T cell proliferative response in vivo and in vitro. The CD4<sup>high</sup> population also expresses memory/activation markers that are consistent with recently activated cells. The finding that Ag-specific T cells up-regulate CD4 expression should aid studies on the dynamics and heterogeneity of the T cell immune response in vivo, the study of suboptimal or heterogenous immune responses, and the identification of T cells that are reactive to known and unknown autoantigens.

## Materials and Methods

### Mice

DBA/2 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in the Stanford Medical Center Department of Comparative Medicine. Mice were used between 8 and 30 wk of age.

### Ag proliferation assays

Groups of three to five mice were immunized at the base of the tail with 100  $\mu$ l of an emulsion containing both IFA plus 10 mg/ml heat-killed *Mycobacterium tuberculosis* and H37RA (Difco Laboratories, Detroit, MI) plus 100  $\mu$ g peptide suspended in an equal volume of Dulbecco's PBS. The peptide SWM(110–121) (AIIHVLHSRHPG) was synthesized and HPLC-purified at the Protein and Nucleic Acid Facility (Beckman Center, Stanford University). At 8 to 10 days postimmunization, draining inguinal lymph node cells were removed, and single-cell suspensions were prepared. A total of  $5 \times 10^5$  cells were incubated in 96-well flat-bottom plates in either T cell media alone or with Ag for whole lymph node proliferation assays. T cell media consisted of RPMI 1640 supplemented with 2 mM L-glutamine, penicillin/streptomycin, nonessential amino acids, sodium pyruvate, and 10 mM HEPES buffer (Life Technologies, Grand Island, NY); 50 mM 2-ME (Sigma, St. Louis, MO); and either 0.5% normal mouse serum or 5 to 10% FCS. Cells were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine after 72 h at 37°C with 6% CO<sub>2</sub> and subsequently harvested after 18 h for counting on a beta plate (Wallac, Gaithersburg, MD).

### FACS analysis

For studies in vitro, aliquots of lymph node cells were taken from culture, washed with FACS buffer (Dulbecco's PBS with 2% FCS), stained for two-color flow cytometric analysis with fluorescein and phycoerythrin Abs at a predetermined optimal concentration for 20 min at 4°C, washed, and stained with propidium iodide (PI) (Sigma) just before analysis. Anti-CD45RB, anti-CD44, anti-CD69, and anti-V $\beta$ 8 fluoresceinated Abs were

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<sup>3</sup> Abbreviations used in this paper: SWM, sperm whale myoglobin; LDA, limiting dilution analysis; PI, propidium iodide.

obtained from PharMingen (San Diego, CA), as was anti-Mel-14 biotinylated Ab. Anti-CD4 and streptavidin-phycoerythrin Abs were obtained from Caltag (San Francisco, CA). A total of  $1 \times 10^5$  cells were analyzed by two-color flow cytometry on a Becton Dickinson FACScan cytometer (Mountain View, CA). The data were analyzed using the Herzenberg desk facility plus Flowjo (Tree Star, San Carlos, CA) on a Power Macintosh (Apple computer, Cupertino, CA). A statistical analysis that included the two-sided Student *t* test was performed with Statview (Abacus Concepts, Berkeley, CA) and Excel (Microsoft, Redmond, WA).  $PI^+$  cells were excluded from analysis.

#### FACS sorting and proliferation assays

Single-cell suspensions were obtained either from 3-day cultures or directly from the lymph nodes of immunized animals (harvested 8–10 days postimmunization in both cases, as described above). The cells were stained with anti-CD4 Ab (in combination with anti-V $\beta$ 8 Ab in some experiments). The cultured cells were analyzed by FACS to determine appropriate gates, sterile-sorted into positive and negative populations, plated into 96-well plates, pulsed with [ $^3$ H]thymidine for 18 h, and then counted on a beta plate. Cells that had been sorted directly from immunized lymph nodes were analyzed, and the highest 1% of cells were selected for sorting; generally 1% analysis levels resulted in an actual sorting of the top ~0.5% of cells. The cells were cultured in 96-well plates at varying amounts (generally between  $1-2 \times 10^4$ ) of  $CD4^{high}$  vs  $CD4^{normal}$  cells; alternately,  $CD4^{high}$  V $\beta$ 8 $^+$  vs  $CD4^{normal}$  V $\beta$ 8 $^+$  cells in 96-well plates containing  $5 \times 10^5$  irradiated syngeneic lymph node or spleen cells with or without Ag were used in the cultures. The cells were then cultured at 72 h before pulsing and harvesting as described above.

#### Limiting dilution analysis (LDA)

Cells that were obtained from culture or directly from immunized mice, were sorted by FACS as described above. After sorting, small aliquots of positive and negative populations were resuspended in FACS buffer and analyzed on the FACS machine that was used for sorting to assess for the percent purity of the sorted populations. The sorted cells were then pelleted and titrated at varying cell numbers into 96-well plates containing irradiated DBA/2 spleen cells, 10  $\mu$ M SWM(110–121), and 10 U/ml IL-2. The plates were cultured for 10 days, at which time the cells were transferred into fresh 96-well plates containing irradiated DBA/2 spleen cells with or without SWM(110–121) and without IL-2. The cells were cultured for an additional 3 days, pulsed with 1  $\mu$ Ci/well [ $^3$ H]thymidine, and harvested for counting after 18 h. The proliferative response was analyzed for responders/nonresponders using four SDs above the response in the absence of Ag as a cutoff for positive responders. Cells were obtained for analysis without Ag in two ways: either some wells in the replicate wells were cultured without Ag, or some wells were split at the time of restimulation and cultured with or without Ag. The proliferative response of  $CD4^{high}$  cells cultured without Ag was consistent across experiments. In some experiments, duplicate plates were cultured at the time of restimulation; one plate was used for the proliferative response, while the second plate was maintained in culture. The percent of negative wells was plotted against cells per plate and analyzed by least-squares linearization using Cricket Graph (Computer Associates, Islandice, NY); the exponential curve-fitting function produced an equation of the form  $y = (\omega) \times 10^{(z)(x)}$ .  $\omega$  and  $z$  were derived from the data, and the resultant precursor frequency ( $x$ ) was calculated by setting  $y = 0.37$  according to Poisson statistics.

## Results

### Ag-specific T cells are $CD4^{high}$

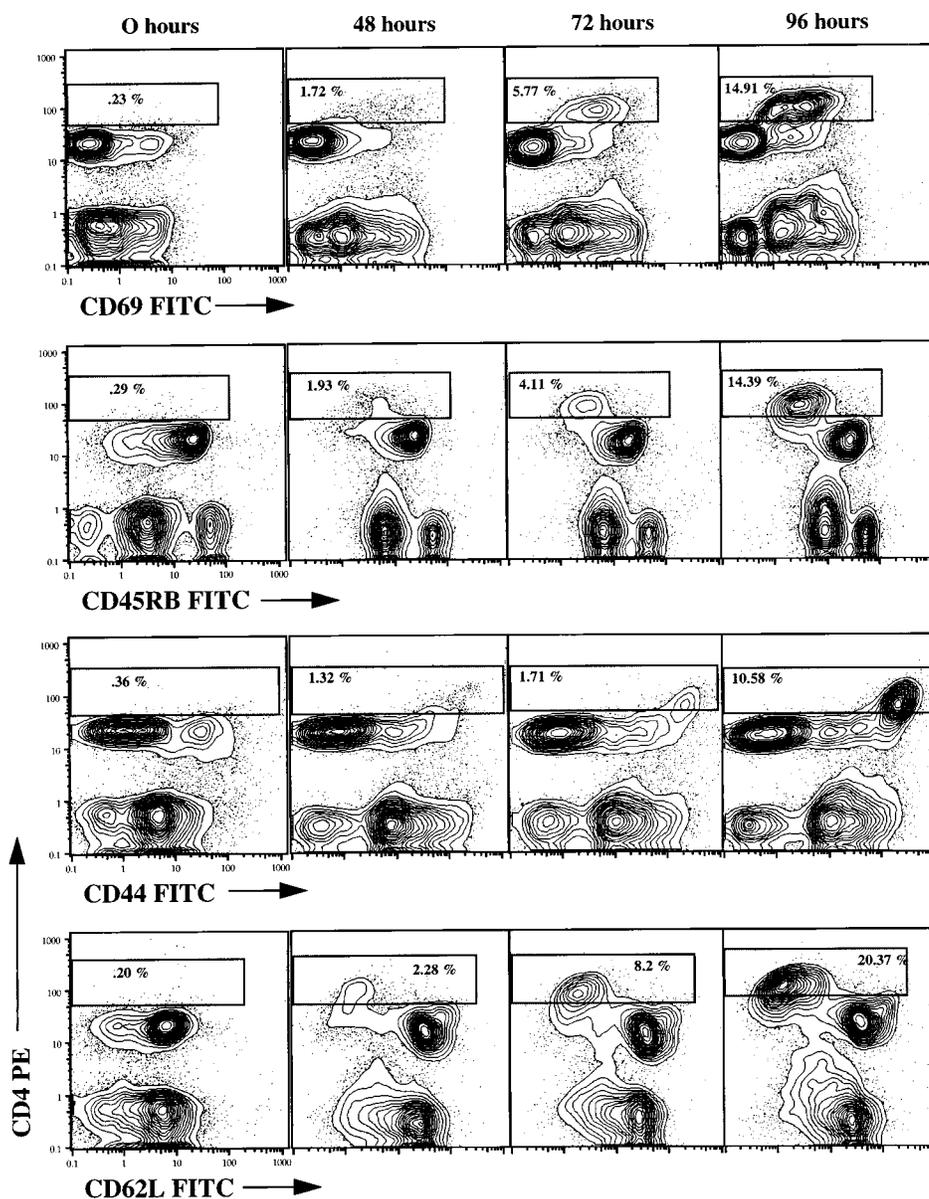
The DBA/2 response to SWM(110–121) that had been previously characterized in our lab (4, 13–15) was initially used to analyze Ag-reactive T cells. Groups of mice were immunized with peptide, and immune lymph node cells were harvested 8 to 10 days later and cultured with or without the immunizing Ag. Periodically, aliquots of cells were taken from culture, stained with Abs to cell surface molecules, and analyzed by flow cytometry for kinetics of expression by live ( $PI^-$ ) cells (Fig. 1). At 48 h, and more obviously at 72 and 96 h, a subpopulation of cells demonstrated increased CD4 expression (Fig. 1). These  $CD4^{high}$  cells were also  $CD45RB^{low}$ ,  $CD62L^{low}$ ,  $CD69^{high}$ , and  $CD44^{high}$  (Fig. 1). T cells from immunized lymph nodes cultured for similar periods of time without Ag showed no comparable  $CD4^{high}$  subpopulation (data not shown). The percentage of  $CD4^{high}$  cells increased steadily

throughout the culture period, from <0.4% of live cells at time 0 to 10 to 20% of cells by 96 h (Fig. 1). The increased percentage was due both to the death of other cells in the culture over time and to an absolute increase in the  $CD4^{high}$  cell numbers (data not shown). A statistical analysis of the  $CD4^{high}$  levels of 13 separate experiments at 72 h postimmunization (the usual time of pulsing in proliferation assays) showed that the difference in CD4 expression between  $CD4^{high}$  and  $CD4^{normal}$  was highly significant (unpaired Student's *t* test,  $p < 0.00001$ ). While there was some variance in the absolute level of CD4 expression in the experiments, the ratio of the  $CD4^{high}$  to  $CD4^{normal}$  was remarkably consistent over all experiments at ~3.5:1. The mean ratios of CD45RB and CD44 in the  $CD4^{high}$  population were also significantly different from the  $CD4^{normal}$  population. The CD45RB expression in the  $CD4^{high}$  group was approximately one-half of the  $CD4^{normal}$  group. The  $CD4^{normal}$  population showed two subpopulations; one expressed ~165-fold less CD44 than the  $CD4^{high}$  cells, while the other expressed ~3-fold less CD44 than the  $CD4^{high}$  population (Fig. 1).

The finding that the  $CD4^{high}$  cells in culture expressed memory/activation markers suggested that they might be the Ag-reactive T cells. To test this possibility, we sorted the  $CD4^{high}$  and  $CD4^{normal}$  cells by FACS after 72 h in culture; the distinct populations seen on FACS analysis (Fig. 1) were used to establish sorting gates. When the cells were sorted and pulsed with [ $^3$ H]thymidine, the  $CD4^{high}$  population contained all of the proliferating cells from the whole lymph node preparation, while the  $CD4^{normal}$  cells did not proliferate above background (Fig. 2B). The whole lymph node cell response to Ag from the same culture was used as a control (Fig. 2A). Thus, all of the Ag-specific proliferative response seen in vitro in response to SWM(110–121) was contained in the  $CD4^{high}$  cell population.

### LDA of $CD4^{high}$ T cells in vitro

It was possible that only a small percentage of the  $CD4^{high}$  cells was actually proliferating in the sorting experiment shown in Figure 2. Thus, we used LDA to quantify the enrichment of Ag reactivity in the  $CD4^{high}$  population. SWM immune DBA/2 lymph node cells were cultured with Ag for 3 days and sorted into  $CD4^{high}$  and  $CD4^{normal}$  subpopulations and then plated at titrated numbers onto irradiated DBA/2 spleen cells in the presence of Ag and IL-2. After 10 days of culture, the cells were transferred to plates with fresh Ag and APCs in the absence of IL-2 and pulsed for a proliferative response after another 3 days of culture. The fraction of negative wells was calculated, allowing for the determination of Ag-specific T cell frequency according to Poisson statistics (Fig. 3). The results presented in Figure 3 demonstrate that the  $CD4^{high}$  subpopulation from the culture contained a substantial enrichment of the Ag-responsive cells; 1 in 5.1  $CD4^{high}$  cells were Ag-reactive compared with typical estimates of Ag frequency in immunized lymph nodes from 1:1000 to 1:10,000 whole lymph node cells (1–3) and compared with the estimate of whole SWM-reactive T cells in primed DBA/2 mice of 1:11,625 that was published previously by our lab (4). The mean of five LDA experiments indicated that 1 in 8.2  $CD4^{high}$  cells was Ag-specific. While the  $CD4^{normal}$  curve was not entirely horizontal (Fig. 3), the  $CD4^{normal}$  cells showed no Ag reactivity after pulsing directly after harvesting from the immunized lymph node (Fig. 2B) or after restimulation with Ag for 3 days before pulsing (Fig. 4). A post-sort analysis established that the residual reactivity seen in the LDA experiments was due to a minor population (<1.5%) of  $CD4^{high}$  cells that contaminated the  $CD4^{normal}$  group during FACS sorting (data not shown).



**FIGURE 1.** Expansion kinetics of CD4<sup>high</sup> T cells during culture. Lymph node cells from DBA/2 mice immunized with SWM(110–121) were cultured with or without Ag (see *Materials and Methods*) and analyzed periodically by FACS for CD4 and CD44, CD62L, CD69, and CD45RB expression. Dead cells were excluded by PI gating. The CD4<sup>high</sup> population is indicated by box outlines; numbers reflect percentages of remaining live cells. The CD4<sup>high</sup> population expresses typical activation/memory markers; CD44<sup>high</sup>, CD69<sup>high</sup>, CD62L<sup>low</sup>, and CD45RB<sup>low</sup> are shown. One representative experiment of five is shown.

#### Ag-specific CD4<sup>high</sup> T cells are present in immune lymph node cells in vivo

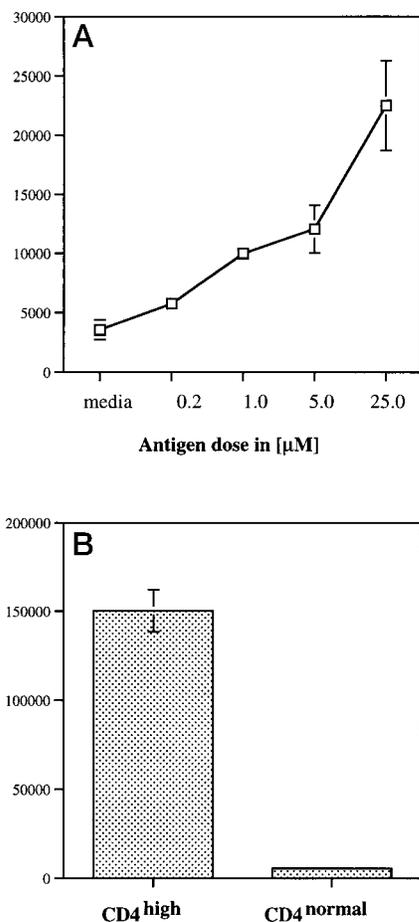
These results demonstrated that the CD4<sup>high</sup> cells contained Ag-responsive cells in vitro. An adaptation of this method to studies in vivo was then attempted. FACS analysis of freshly harvested lymph node cells from DBA/2 mice (taken at various time points after immunization with SWM(110–121)) showed no obvious expansion of CD4<sup>high</sup> cells (see Fig. 5A, top panel, compared with Fig. 1). We reasoned that the Ag-reactive CD4<sup>high</sup> T cell population was present but had not expanded as dramatically in vivo as in vitro. To test this hypothesis, we again used the DBA/2 response to SWM(110–121), which shows a predominant V $\beta$ 8 response when cells are cloned and T cell hybridomas are made (13–15). We sorted CD4<sup>high</sup> V $\beta$ 8<sup>+</sup> T cells directly from the lymph nodes of SWM-immunized DBA/2 mice; gates set to select the highest CD4 expressing V $\beta$ 8<sup>+</sup> T cells (representing ~0.5% of lymph node cells) were used, and those cells, vs CD4<sup>normal</sup> V $\beta$ 8<sup>+</sup> T cells, were cultured with naive, irradiated DBA/2 spleen cells with or without Ag (the lymph node T cell proliferative response from the same mice served as control) (Fig. 4). The CD4<sup>high</sup> V $\beta$ 8<sup>+</sup> T cells demonstrated a robust Ag proliferative response, while the CD4<sup>normal</sup>

V $\beta$ 8<sup>+</sup> T cells did not. These experiments demonstrated that the Ag-reactive T cell CD4<sup>high</sup> subpopulation could be identified in vivo.

#### LDA of CD4<sup>high</sup> T cells activated in vivo

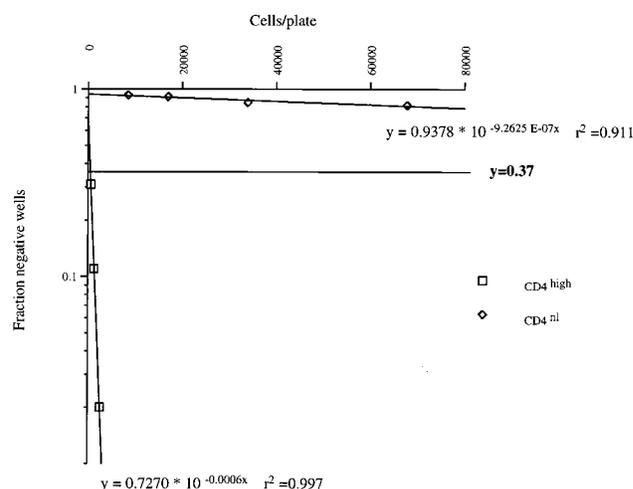
Next, we quantified the enrichment of the CD4<sup>high</sup> Ag-specific T cell precursor frequency of CD4<sup>high</sup> T cells activated in vivo using LDA. We sorted CD4<sup>high</sup> V $\beta$ 8<sup>+</sup> T cells directly from freshly harvested SWM(110–121)-immunized lymph nodes at 8 days postimmunization (Fig. 5A); the top ~1% of CD4 brightness in the CD4<sup>+</sup> V $\beta$ 8<sup>+</sup> cell population was used as a sorting gate (Fig. 5A). We cultured titrated, sorted cell numbers in 96-well plates with naive, irradiated DBA/2 spleen cells, Ag, and IL-2, and restimulated the cultures at 10 days. We consistently found a substantial enrichment of Ag reactivity in the V $\beta$ 8<sup>+</sup> response (1 in 32.4 CD4<sup>high</sup> cells, Fig. 5B) in the 8 day in vivo SWM(110–121) response. The result was quite consistent over five LDA experiments, with a mean indicating that 1/32.8 CD4<sup>high</sup> cells was Ag-specific from primed lymph nodes in vivo.

The frequency of CD4<sup>high</sup> V $\beta$ 8<sup>+</sup> cells for SWM(110–121) in vivo was lower than that seen in the in vitro analysis (Fig. 3). The



**FIGURE 2.** The proliferative response to Ag is contained within the CD4<sup>high</sup> population in culture. SWM(110–121) primed lymph node cells were cultured as described in *Materials and Methods*. After 3 days, the response of 500,000 whole lymph node cells was assayed by [ $^3\text{H}$ ]thymidine incorporation (A), while a separate aliquot of cells was sterile-sorted by flow cytometry into CD4<sup>high</sup> and CD4<sup>normal</sup> populations. A total of  $7.5 \times 10^4$  cells were plated and pulsed immediately with [ $^3\text{H}$ ]thymidine for 18 h before counting on a beta plate (B). Error bars are the SEM; one representative experiment of five is shown.

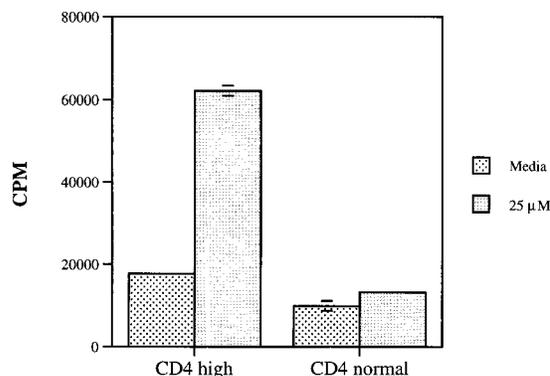
lower precursor frequency demonstrated in vivo compared with studies in vitro could be attributed in part to the technical difficulty of extracting cells directly from the mouse and sorting/culturing them for prolonged periods. In addition, an analysis of the response of the CD4<sup>high</sup> T cells to purified protein derivative demonstrated that some CD4<sup>high</sup> cells, when taken directly from immunized mice, were primed by the purified protein derivative contained in the CFA that was used as adjuvant (data not shown). CFA-primed cells do not expand during culture with SWM; hence, the CD4<sup>high</sup> population in vitro is further enriched for SWM-reactive cells at the time of cell sorting from culture. While the CFA primed cells do not expand during restimulation in vitro with SWM, they will dilute the frequency of CD4<sup>high</sup> cells that recognize SWM at the time of sorting (sorting is performed before expansion with the in vivo-primed cells). A final explanation of the fourfold less enrichment in vivo compared with the in vitro sorting of CD4<sup>high</sup> is suggested by a comparison of the empiric sort gates chosen in vivo (Fig. 5A) with the gates established in vitro (Fig. 1). The consistent mean ratio of CD4<sup>high</sup> to CD4<sup>normal</sup> expression in vitro suggested that the gates chosen in vivo allowed a significant number of CD4<sup>normal</sup> cells into the “CD4<sup>high</sup>” population.



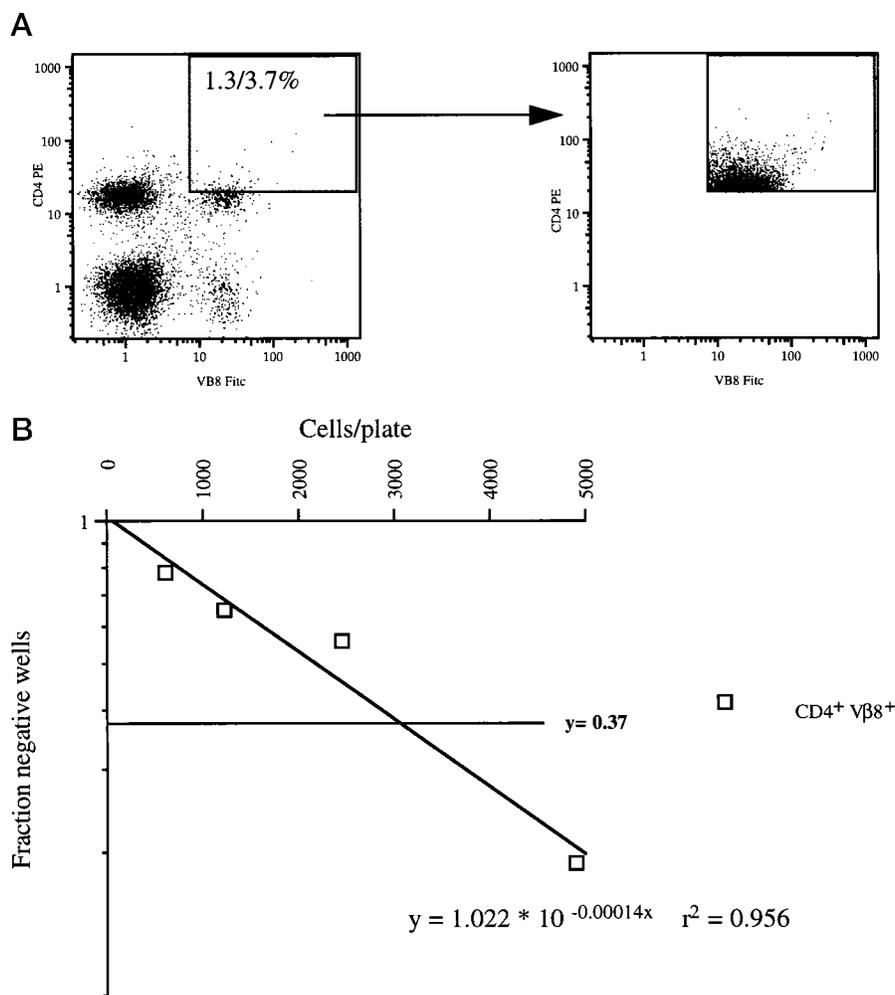
**FIGURE 3.** LDA quantitates the precursor frequency of CD4<sup>high</sup> cells. SWM(110–121) primed lymph node cells were cultured and sorted as described in Figure 2. Titrated numbers of sorted CD4<sup>high</sup> and CD4<sup>normal</sup> cells were added to 96-well plates with irradiated spleen cells and Ag for culture (see *Materials and Methods*). The number of cells per plate that averaged 1 Ag-reactive cell/well was calculated by Poisson statistics. A total of 1 in 5.1 CD4<sup>high</sup> cells were Ag-specific in this experiment. One representative experiment of five is shown.

We have demonstrated that T cells from all mouse strains studied to date (following immunization with peptides or proteins) up-regulate CD4 expression in response to Ag; e.g., PLJ  $\times$  SJL)F<sub>1</sub> mice in response to myelin basic protein (unpublished observations), nonobese diabetic mice in response to self and foreign peptides, and naive hen egg lysozyme(46–61)-responsive transgenic T cells in culture (our unpublished observations). We postulate that the up-regulation of CD4 in response to peptide Ags is a universal T cell response to initial confrontation with “nominal” Ag in vivo. CD4<sup>high</sup> T cells contain a heterogeneous population of responding T cells.

Next, we investigated whether CD4<sup>high</sup> expression could be used as a method of identifying and characterizing those Ag-specific cells in vivo that are not normally expanded under competitive culture conditions (i.e., suboptimal T cell proliferative responses). The CD4<sup>high</sup> approach allowed for the possibility of



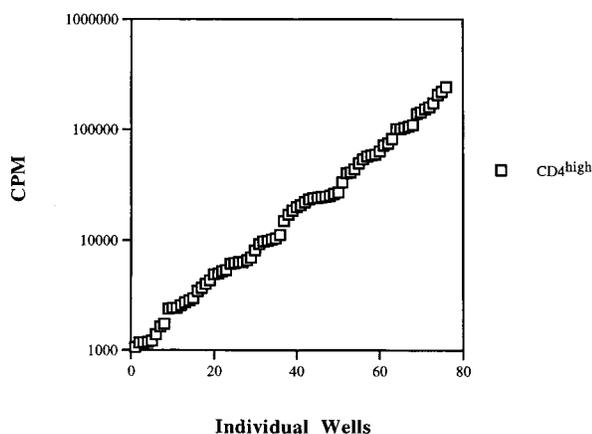
**FIGURE 4.** CD4<sup>high</sup> cells contain Ag-reactive cells in vivo. Freshly harvested SWM(110–121)-immunized DBA/2 lymph node cells were stained and sorted for  $1.2 \times 10^4$  CD4<sup>high</sup> V $\beta$ 8<sup>+</sup> ( $\leq 0.5\%$  of lymph node cells) or CD4<sup>normal</sup> V $\beta$ 8<sup>+</sup> cells, which were then cultured for 3 days with irradiated DBA/2 spleen cells plus SWM(110–121) before pulsing with [ $^3\text{H}$ ]thymidine. Error bars represent the SEM; one representative experiment of at least three is shown.



**FIGURE 5.** LDA of  $CD4^{\text{high}}$  cells allows for the quantification of immune response kinetics in vivo.  $CD4^{\text{high}} V\beta 8^+$  cells were sorted directly from freshly harvested, SWM(110–121)-primed lymph nodes as described in Figure 4 (A). The  $CD4^+$   $V\beta 8^+$  population was 15% of total  $CD4^+$  cells in the presorted lymph node (A). The gates were determined as the brightest 1.3% of all lymph node cells from the  $CD4^+$   $V\beta 8^+$  subset by presort analysis, which represented the brightest 3.7% of  $CD4$  cells from that subset. A shows the FACS plot of the immunized lymph node and data on  $5 \times 10^3$  cells from the sorting subset that were collected during the sort. The cells were plated and restimulated (see *Materials and Methods*), and the precursor frequency was calculated by linear curve-fitting according to Poisson statistics (B). One representative experiment of five is shown.

individually expanding suboptimally reactive T cells directly from the immunized lymph node. To accomplish this expansion, LDAs were conducted as described above, and  $CD4^{\text{high}} V\beta 8^+$  cells were plated into replicate plates at the time of restimulation. One of the two plates was then cultured for 3 additional days and pulsed with [ $^3\text{H}$ ]thymidine to establish the dilution of cells at which one cell or less was present in the well. The proliferative responses of individual positive wells grown out from initial conditions at  $\sim 1$  cell/well (Fig. 6) (as calculated by the LDA from Poisson statistics) were plotted. The responses of positive wells expanded under identical culture conditions from individual  $CD4^{\text{high}} V\beta 8^+$  cells showed a continuous distribution (over  $\sim 3$  logs of cpm) of proliferative responses from very high responders to very low responders (Fig. 6). This distribution stands in contrast to the expected behavior of a clonal population, which should show a normal distribution of proliferative responses (i.e., most of the response occurring near a mean, with some outliers on each end). The uncloned  $CD4^{\text{high}}$  cells from immune lymph nodes, on the contrary, show equal numbers of cells proliferating at every level (Fig. 6). It is natural to speculate that if the fast-growing  $CD4^{\text{high}}$  cells (as plotted in Fig. 6, *far right*) were cultured under standard cloning conditions with slow-growing  $CD4^{\text{high}}$  T cells (Fig. 6,

*left*), the fast cells would outgrow the slower cells. The advantage of this limiting dilution method of cloning cells directly from the lymph node is that the separate cells, when cultured at one cell (or less) per well, can be expanded without the pressure of competitive growth, thus enabling a study of suboptimal responders to Ag in vivo. There are two likely explanations for the unequal growth of responding  $CD4^{\text{high}}$  cells plated under identical conditions. One possibility is that an unknown stimulus continuously distributed under the conditions of culture causes T cells with inherently equal Ag responsiveness to proliferate unequally. The second explanation is that the responding T cells display a continuous distribution of growth (proliferation) in response to a single Ag concentration. If the growth response reflects an intrinsic difference between the responding T cells, such as TCR affinity for Ag, the phenotype of low vs high responders should persist after repetitive stimulation and rest of the T cells. The system outlined here allowed us to investigate that question. Preliminary results indicate that low responder cells can be cloned (from the replicate plates) and maintained in culture, and not only retain their low proliferative phenotype but also express different (nondominant)  $V\beta$  repertoires from the high responder cells (M.F., manuscript in preparation).



**FIGURE 6.** Rank ordering of the proliferative response of the progeny of single CD4<sup>high</sup> T cells obtained by limiting dilution to fixed Ag dose demonstrates the heterogeneity of the immune response in vivo. After sorting, culturing, and counting the [<sup>3</sup>H]thymidine incorporation of CD4<sup>high</sup> Vβ8<sup>+</sup> cells sorted directly from SWM-immunized DBA/2 mice, an LDA was performed (as in Figs. 3 and 5), and then the proliferative responses of individual positive wells from a plate receiving ~1 Ag-specific cell/well were plotted in rank order. The CD4<sup>high</sup> Ag-reactive T cells demonstrated equal numbers of cells over a wide distribution (~3 logs cpm) of the proliferative response to a fixed Ag dose.

## Discussion

The results presented here demonstrate that the Ag-specific T cells responding to an antigenic challenge in vitro and in vivo are CD4<sup>high</sup> T cells. The CD4<sup>high</sup> population contains all of the T cell proliferative response to Ag in vitro and in vivo (Figs. 2B and 4). Moreover, depleting the CD4<sup>high</sup> T cells from whole lymph node populations that are responsive to the immunizing Ag ablates the immune response (i.e., the CD4<sup>normal</sup> population that remains after depleting the CD4<sup>high</sup> T cells does not proliferate when pulsed (Fig. 2B), nor does it proliferate when cultured with the immunizing Ag (Fig. 4)). An LDA to obtain the precursor frequency of Ag-specific T cells in the CD4<sup>high</sup> populations both in vitro and in vivo (Figs. 3 and 5) demonstrates a substantial (100–1000-fold) enrichment of Ag-specific T cells compared with traditional cloning methods in general (1–3, 5, 16) and with our previous results in the SWM/DBA2 system in particular (4). In addition, the CD4<sup>high</sup> marker can be used to follow the Ag-specific T cell response in vivo (Fig. 5) and allows a method to study T cells that have a suboptimal response to Ag (Fig. 6). The possibility of studying a variety of T cells with different Ag recognition properties while holding the Ag dose constant is a novel approach that bypasses some of the obstacles presented by the traditional method of studying a single, high affinity T cell in response to a varying Ag dose. Since we have demonstrated the up-regulation of CD4 by T cells upon first contact with their nominal Ag in several different Ag systems and in several strains of mice, these findings should have broad applicability in immunology. These results introduce the possibility of using the CD4<sup>high</sup> marker to study a variety of Ag-specific autoimmune T cell responses in vitro and in vivo. For example the study of suboptimal T cell responses to date has used T cell clones that respond well to their nominal Ag but respond suboptimally to different doses of an altered peptide ligand (17–19). The use of the CD4<sup>high</sup> marker allows us to invert this approach; using a constant dose of an Ag, we can derive T cells that respond suboptimally to that Ag in vivo. It has now been well-established that low proliferative responder T cells can manifest Ag responses to altered peptides by alterations in cytokine pro-

duction (17–19). We anticipate that T cells with suboptimal T cell responses might demonstrate an altered cytokine profile compared with T cells that respond with the “usual” high proliferative response to Ag. As another potential application, since the expression of CD4 increases in T cells following their recognition of Ag, and the CD4<sup>high</sup> T cells can be recovered directly from mice, this method can potentially be applied to isolating autoreactive T cells and identifying the autoantigen recognized by the CD4<sup>high</sup> autoreactive T cells (20).

It is somewhat surprising that CD4 up-regulation by T cells in response to their nominal Ag has not been previously reported. Many of the earlier studies of T cell activation used Jurkat cells or T cell hybridomas and stimulated them with less-specific reagents, including lectins, ionomycin, and Abs; in some of these experiments, CD4 reportedly decreased in expression following activation (21–23). In our own lab, we have observed that PMA and ionomycin stimulation can cause an apparent down-regulation of CD4 (our unpublished data). The different outcome of CD4 expression following stimulation with specific Ag vs “stronger” or less-specific T cell-activating agents may reflect a physiologic difference in the mechanism of activation. Studies using T cell clones have also not shown CD4 up-regulation. We derived Ag-specific T cell lines and examined CD4 expression after 10 days of culture; the surviving cells were CD4<sup>high</sup> as expected from Figure 1 (data not shown). We then compared the CD4 levels of an established T cell clone (11.3, derived from DBA/2 mice by immunization with SWM(110–121) followed by alternating rest and activation) at 3 days and at 10 to 14 days following activation; the CD4 levels did not change regardless of the activation state (data not shown). From these experiments, we conclude that T cell clones have already up-regulated CD4 expression, which explains why CD4 up-regulation was not seen in studies involving T cell clones. These results also suggest that CD4<sup>high</sup> may be a persistent phenotype marking memory-like cells; this possibility is supported by LDA experiments showing Ag-specific cells in the CD4<sup>high</sup> group in vivo for ≥4 wk postimmunization (M.F., manuscript in preparation).

Although it was not studied here, it is tempting to speculate upon the biologic significance of CD4 up-regulation in the T cell response to Ag. The role of CD4 as a coreceptor has been persuasively argued by Janeway, who proposed that the association of CD4 with the TCR during the process of T cell activation could allow enhanced signal amplification (24, 25). In contrast, naive T cells showed no association of CD4 with the TCR (26). In the CD4 coreceptor model, the increased CD4 expression we have demonstrated could provide a mechanism for enhanced signal transduction via a stoichiometric increase in the amount of CD4 in the TCR activation complex. Another view of the role of CD4 in TCR signaling has been proposed by Germain and coworkers (27), using a density model of partial agonist signaling. Germain suggested that low vs high densities of CD4 could affect the dissociation time of the TCR with its peptide/MHC complex (27). In the density model, the higher CD4 surface expression that we have demonstrated on activated Ag-specific T cells could enhance signaling capability via an enhancement of overall T cell:MHC/peptide avidity. These and other models of the effect of increased CD4 expression on Ag-specific, activated T cells may be amenable to experimentation using the approach described in this paper.

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