Response of Naive Antigen-specific CD4+ T Cells In Vitro: Characteristics and Antigen-presenting Cell Requirements

By Michael Croft, David D. Duncan, and Susan L. Swain

From the Department of Biology and the Cancer Center, University of California, San Diego, La Jolla, California 92093

Summary

Because of the low frequency of T cells for any particular soluble protein antigen in unprimed animals, the requirements for naive T cell responses in specific antigens have not been clearly delineated and they have been difficult to study in vitro. We have taken advantage of mice transgenic for the Vβ3/Vα11 T cell receptor (TCR), which can recognize a peptide of cytochrome *c* presented by IE^k. 85–90% of CD4+ T cells in these mice express the transgenic TCR, and we show that almost all such Vβ3/Vα11 receptor–positive cells have a phenotype characteristic of naive T cells, including expression of high levels of CD45RB, high levels of L-selectin (Mel-14), low levels of CD44 (Pgp-1), and secretion of interleukin 2 (IL-2) as the major cytokine. Naive T cells, separated on the basis of CD45RB high expression, gave vigorous responses (proliferation and IL-2 secretion) to peptide antigen presented in vitro by a mixed antigen-presenting cell population. At least 50% of the T cell population appeared to respond, as assessed by blast transformation, entry into G_1, and expression of increased levels of CD44 by 24 h. Significant contributions to the response by contaminating memory CD4+ cells were ruled out by demonstrating that the majority of the CD45RB low, L-selectin low, CD44 high cells did not express the Vβ3/Vα11 TCR and responded poorly to antigen. We find that proliferation and IL-2 secretion of the naive CD4 cells is minimal when resting B cells present peptide antigen, and that both splenic and bone marrow–derived macrophages are weak stimulators. Naive T cells did respond well to high numbers of activated B cells. However, dendritic cells were the most potent stimulators of proliferation and IL-2 secretion at low cell numbers, and were far superior inducers of IL-2 at higher numbers. These studies establish that naive CD4 T cells can respond vigorously to soluble antigen and indicate that maximal stimulation can be achieved by presentation of antigen on dendritic cells. This model should prove very useful in further investigations of activation requirements and functional characteristics of naive helper T cells.

Naive, immunocompetent, CD4+ T cells play a central role in the generation of the B cell and the cytolytic T cell response, and are essential for responses to new foreign antigens. Although naive CD4 cells produce IL-2 as their only major cytokine and possess little helper activity for B cells (reviewed in reference 1), they can differentiate, over a period of 3–5 d, into a population of primary effector cells that are capable of producing the full array of cytokines (e.g., IL-2, IL-4, IL-5, IFN-γ, IL-6, etc.), and are extremely efficient helpers of B cell responses (2–5). Effectors are generated both in vitro in responses to mitogenic stimulation or specific antigen stimulation (1–3), and in vivo in responses to protein antigen stimulation (4).

However, because of low numbers of naive T cells specific for any particular protein antigen, in vitro studies of naive T response have been limited to studying cells of naive phenotype, usually CD45RA or RB high, or CD44 or LFA-3 low, stimulated with mitogens or alloantigens. These polyclonal stimulants provide levels of receptor crosslinking that may be much greater than those achieved in physiologic responses to noncellular antigens, and the heterogeneity of the isolated subpopulations suggests that they are unlikely to represent "pure" naive T cells (discussed in reference 6). Surprisingly, the reports with these models have often concluded that naive CD4 T cells respond rather poorly, especially in comparison with cells of memory phenotype (reviewed in reference 6). Therefore, the requirements for antigen-specific stimulation of naive helper T cells are unclear.

Many cell types have been implicated as APC for unseparated T cell populations and T cell clones. A series of definitive studies over the past 15 years by Steinman and colleagues (reviewed in reference 7) have shown that dendritic cells are far more potent stimulators of T cells than other class II–expressing cells such as macrophages and B cells. Most reports

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analyzing the APC requirements for T cell activation utilized T cells from unprimed mice, which consist of a mixture of naive and memory cells. When taken from young individuals such cells have a predominantly naive phenotype, and it has been suggested that dendritic cells are the major APC for naive T cells (7–9).

To directly assess the response of naive CD4 T cells, we have recently developed an in vitro model using mice, from Kaye et al. (10), that are transgenic for a Vα11/Vβ3 TCR recognizing a peptide of cytochrome *c* (pigeon cytochrome *c* fragment [PCCF][1]) presented on IE^k-bearing APC (10). In these mice, at least 80% of the T cells are $CD4^+$ and lymph node cells have been shown to proliferate to PCCF presented on an APC population (10). We reasoned that the majority of transgene-expressing CD4 T cells from unprimed mice should never have encountered the specific antigen, PCCF, and that CD4 cells from these animals with naive phenotype should provide an excellent source of bona fide naive cells for experimentation. Our results confirm that most transgene-expressing CD4 T cells are indeed naive, and demonstrate that the naive helper cells make impressive responses to PCCF presented on large, activated B cells and on dendritic cells.

Materials and Methods

Mice. $H\text{-}2^b$ TCR α/β transgenic mice were derived by mating a (C57BL6 × SJL)F_1 male (10), kindly provided by Dr. S. Hedrick (University of California, San Diego, La Jolla, CA) to C57BL6 females with successive backcrosses to a C57BL6 background. Transgenic ($H\text{-}2^{b/b}$) males backcrossed three to five times to C57BL6 were bred to B10.Br females to produce transgenic $H\text{-}2^{b/k}$ offspring, which were maintained at the animal facilities at the University of California, San Diego, and used at 2–4 mo of age.

Cell Lines and Antibodies. Cell lines and Ab used for assays and/or cultures have been described previously (2–5). All cell lines and cell line supernatants were free from *Mycoplasma* and endotoxin contamination. Ab used for T cell depletion were anti-Thy-1.2 (F7D5 and HO.13.14), anti-L3T4 (RL172.4), and anti-Ly-2.2 (HO.2.2). Ab used for staining cell populations were rat anti-mouse Ab to CD45RB (23G2; a gift from E. Puré, The Rockefeller University, New York, NY), L-selectin/$gp90^{mel}$ (Mel-14; from I. L. Weissman, Stanford University, Stanford, CA), CD44 (Pgp-1; a gift from I. Trowbridge, The Salk Institute, San Diego, CA), Vα11 (Pharmingen, San Diego, CA), CD4 (GK1.5; Becton Dickinson & Co., Mountain View, CA), B220 (Pharmingen), Mac-1 (American Type Culture Collection, Rockville, MD), 33D1 (a gift from R. M. Steinman, The Rockefeller University), and mouse anti-rat κ (RG79.1; from American Type Culture Collection), and hamster anti-mouse Vβ3 (KJ25; a kind gift from Dr. J. W. Kappler, Howard Hughes Medical Institute, National Jewish Center, Denver, CO).

T Cells. Purified $CD4^+$ T cells were isolated as previously described (2–5). Briefly, spleen cells from transgenic $H2^{b/k}$ mice were passed over nylon columns and treated with anti-CD8 (HO.2.2) and anti-HSA (J11D) plus complement. Separation of CD4 T cells into CD45RB high and low populations was accomplished using magnetic columns (MACS; Miltenyi Biotec, Sunnyvale, CA) as previously described (4). Purified cells were labeled sequentially with rat anti-mouse CD45RB (23G2), biotinylated mouse anti-rat κ (RG7), FITC-streptavidin (Zymed, San Francisco, CA), and biotinylated magnetic beads. Positively selected cells were >95% $CD4^+$ and >90% CD45RB high. Negatively selected cells were >95% $CD4^+$ and 80–90% CD45RB low. Purities of separated CD4 T populations are shown in Fig. 1.

APC. Dendritic cells, splenic macrophages, and activated B cells were prepared 1 d before T cells and resting B cells were prepared the same day as the T cells. Cultures for obtaining bone marrow–derived macrophages (BMDM) were initiated 1 wk before. All APC were from (C57BL6 × B10.Br)F_1 mice. The phenotype and purity of the populations are shown in Fig. 2.

Dendritic Cells. Dendritic cells were prepared using a modification of the method originally described by Steinman and Witmer (11). Collagenase-treated spleen cells (1 μg/ml, 30 min, 37°C) were spun on a discontinuous percoll gradient (three layers, 45%, 60% and 80% [1.068, 1.080, 1.095 kg/liter, respectively]). Cells from the medium/45% and 45%/60% interfaces were pooled, washed, and resuspended in RPMI. Macrophage and dendritic cells were enriched by two rounds of adherence in 60-mm petri dishes (Falcon Labware, Oxnard, CA) at 37°C for 2 h. After an overnight incubation, the floating detached cells, highly enriched in dendritic cells, were harvested. Contaminating macrophages in the dendritic population were further depleted by an additional 2-h readherence step.

Splenic Macrophages. A macrophage-enriched population was obtained by the same overnight adherence procedure used to purify dendritic cells. In this case, nonadherent cells were removed and adherent cells isolated by scraping. This macrophage-enriched population was readhered for a further 2–3 h and the remaining adherent cells were again removed by scraping and used as the macrophage population.

Activated Large B Cells. High-density spleen cells were collected from the 60%/80% percoll interface (above) and were treated with anti-T cell Ab plus complement. Cells were then adhered for 2 h on plastic. The resultant nonadherent, dense B cells (>95% $B220^+$, small cells, as judged by staining and forward scatter; data not shown) were stimulated at 2×10^6/ml with 0.1 μM phorbol dibutyrate and 0.1 μg/ml ionomycin. After overnight incubation, cells were separated on a four-layer percoll gradient (40%, 53%, 62%, 80%) and low-density cells were collected from the 40%/53% interface. The low-density population was depleted of any adherent cells and used as the activated B cell population.

Resting Small B Cells. Spleen cells were adhered for 1 h in plastic flasks and the nonadherent cells collected and treated with anti-T cell Ab and complement. The T-depleted cells were separated on a four-layer Percoll gradient (as for activated B cells, above) and high-density cells recovered from the 62%/80% interface. Dense cells were again depleted of any adherent cells.

BMDM. Bone marrow was removed from femurs by expulsion with medium. Washed cells were resuspended in DMEM (Gibco Laboratories, Grand Island, NY) supplemented with 10% CSF-1 containing L cell supernatant and grown for 6 d in plastic flasks. They were then washed several times and recultured overnight in fresh DMEM containing 100 U/ml IFN-γ to induce reexpression of class II MHC. After induction, cells were then removed by scraping, and washed several times. BMDM were >99% $Mac\text{-}1^+$, and expressed undetectable levels of 33D1 and B220 (data not shown).

Cell Cultures. Cells were cultured in RPMI 1640 (Gibco Laboratories) supplemented with penicillin (200 μg/ml), streptomycin (200 μg/ml), glutamine (4 mM), 2-ME (50 μM), sodium pyruvate (1 mM), HEPES (10 μM), and 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT). Cells were cultured in 100-μl volumes

[1] *Abbreviations used in this paper:* BMDM, bone marrow–derived macrophage; PCCF, pigeon cytochrome *c* fragment.

in tilted flat-bottomed 96-well plates (Costar, Cambridge, MA). Proliferation was assessed after 48, 72, or 96 h of culture with 1 μCi [^{3}H]thymidine added during the last 16 h of culture. Cells were harvested on a PHD cell harvester (Cambridge Technology, Atlantic Beach, NY), and incorporated radioactivity was assessed on a scintillation counter. IL-2 secretion was measured in supernatants recovered after 24–36 h of culture, using the NK bioassay in the presence of anti-IL-4, as previously described (2–5).

Fluorescence Analysis. Dendritic cells and macrophages were visualized with 33D1 and Mac-1, respectively, followed by fluoresceinated RG7 (mouse anti-rat κ chain). B cells were visualized by staining with PE-conjugated B220. Nonspecific binding to Fc receptors was avoided by preincubation of cells in normal mouse serum. Isotype-matched rat antibodies were used as negative controls for staining, and dead cells were gated out by excluding those that stained with propidium iodide. CD4 T cells were stained as previously described (2, 4). T cell populations were examined using three-color staining. First, unlabeled Mel-14 (L-selectin) or Pgp-1 (CD44) were followed by FITC-RG7, or unlabeled KJ25 (Vβ3) was followed by FITC-goat anti-hamster Ig. Cells were washed, and PE-GK1.5 was used to stain CD4. Final staining after washing was done with biotinylated 23G2 (CD45RB) and Tricolor-streptavidin (Caltag Laboratories, San Francisco, CA). Cell populations were examined on a FACScan® analyzer (Becton Dickinson & Co. computer program).

Results and Discussion

The Majority of Transgene-expressing CD4 T Cells Have a Naive Phenotype. The purity and cell surface phenotypes of representative T cell populations studied are shown in Fig. 1. Fig. 1 *a* shows the profile of CD45RB expression on CD4 cells in the transgenic mice. Approximately 90% of CD4$^+$ cells expressed high levels of CD45RB. Analysis of gated CD4$^+$, CD45RB high expressing cells (right of dotted line in Fig. 1 *a*) by three-color staining (Fig. 1, *c* and *d*, *solid line*) revealed that nearly all the CD45RB high cells expressed low

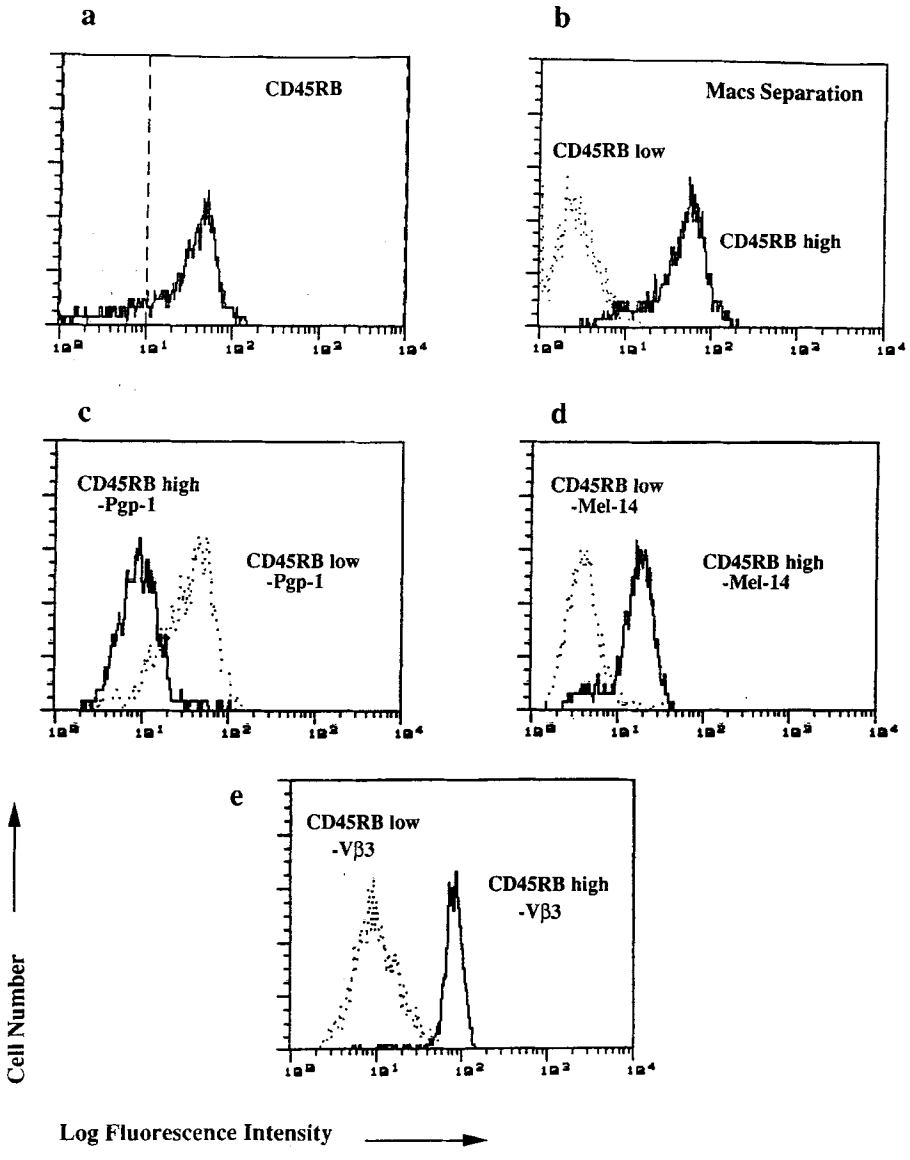

Figure 1. Surface marker expression of transgenic CD4$^+$ T cells. Three-color staining was performed as described in Materials and Methods. The expression of CD45RB by CD4$^+$ T cells and of other markers, including CD44 (Pgp-1), L-selectin (Mel-14), and Vβ3, on CD45RB high and low populations is shown. (*a*) Expression of CD45RB on CD4$^+$ cells, and (*c*) Pgp-1, (*d*) Mel-14, (*e*) and Vβ3 expression on CD4$^+$, CD45RB high cells (*solid lines*) or CD4$^+$, CD45RB low cells (*dotted lines*). For experiments, CD4$^+$, CD45RB high naive cells and CD4$^+$, CD45RB low memory cells were separated on magnetic columns as shown *b*.

levels of Pgp-1/CD44 and high levels of Mel-14/L-selectin, two other markers previously shown to distinguish naive from memory cells (12–14). CD45RB low cells (Fig. 1, *c* and *d*, *dotted line*) had a reciprocal phenotype. In addition, CD45RB low cells expressed higher levels of LFA-1 and ICAM-1 than CD45RB high cells (data not shown). Thus, the CD45RB high cells had a naive phenotype and CD45RB low cells a memory phenotype by each criterion. Importantly, >99% of the $CD4^+$, CD45RB high cells expressed the transgenic TCR as shown by $V\beta3$ staining (Fig. 1 *e*, *solid line*) and $V\alpha11$ staining (not shown). Few CD45RB low cells (of putative memory phenotype) expressed $V\beta3$ (Fig. 1 *e*, *dotted line*), or $V\alpha11$ (not shown). This suggests that cells selectively expressing endogenous TCR chains are the only ones to have responded to the antigens in the environment and have been preferentially driven into the memory cell pool. Fig. 1 *b* shows CD45RB high and low CD4 T cells from a representative separation using magnetic beads. The purity of the naive cells was repeatedly 90–95% and of the memory cells was 80–90%. Thus, the CD45RB high cells represent an unusually homogeneous population of naive CD4 cells of single antigen specificity.

Characterization of APC Populations. Fig. 2 shows the phenotype profiles of representative populations of splenic macrophage, dendritic cell, activated B cell, and resting B cells. Cell populations were stained for the lymphoid dendritic cell marker, 33D1 (15), the macrophage marker, Mac-1, and the B cell marker, B220. Splenic macrophages were ~85–90% $Mac\text{-}1^+$, ~10–15% $33D1^+$ with no detectable $B220^+$ cells. Dendritic cells were 60–80% pure ($33D1^+$) contaminated only by macrophages (no detectable $B220^+$ cells). Both resting and activated B cell populations expressed no detectable 33D1 or Mac-1, and they were >99% $B220^+$. Activated B cells had much higher levels of forward scatter than resting B cells (not shown). BMDM were also used in some experiments and consisted of >99% $Mac\text{-}1^+$ cells (data not shown).

Naive $CD4^+$ T Cells Respond Vigorously to Antigen by Proliferating and Secreting IL-2. CD45RB high and low cells were titrated onto a fixed number of unseparated mitomycin-treated T-depleted splenic APC in the presence of PCCF, and T cell proliferation was measured at various times along with IL-2 secretion after 24–36 h. Maximum proliferation occurred at 48–72 h and dropped off at later time points. Fig. 3 *a* shows proliferation at 48–72 h and IL-2 titers on the supernatants of 2.5×10^4 T cells to an equal number of APC in the presence of varying concentrations of antigen. Maximal proliferation was obtained with ~5 μM PCCF. IL-2 secretion was dependent on antigen concentration with a similar profile. In subsequent experiments we used 10 μM PCCF to ensure that antigen was not limiting. T cell proliferation and IL-2 secretion varied with T cell number when a fixed concentration of APC (5×10^4) and 10 μM PCCF was added (Fig. 3 *b*). Therefore, we chose a T cell number of 2.5×10^4 per culture for subsequent experiments, which should have given an easily detectable but still T cell dose–dependent response. The CD45RB high cells (Fig. 3, *open squares*) responded extremely well by both proliferation and IL-2 secretion. In contrast, the CD45RB low population responded poorly to PCCF (*filled squares*). This was to be expected since the frequency of transgene-expressing cells was low in that population. The weak response of the CD45RB low population argues forcibly against the possibility that contaminating memory cells in the RB high population could account for the excellent response of those cells to peptide. These results establish that naive cells will proliferate well and secrete substantial titers of IL-2 in response to appropriately presented soluble antigen. While primary responses of "naive" phenotype T cells to

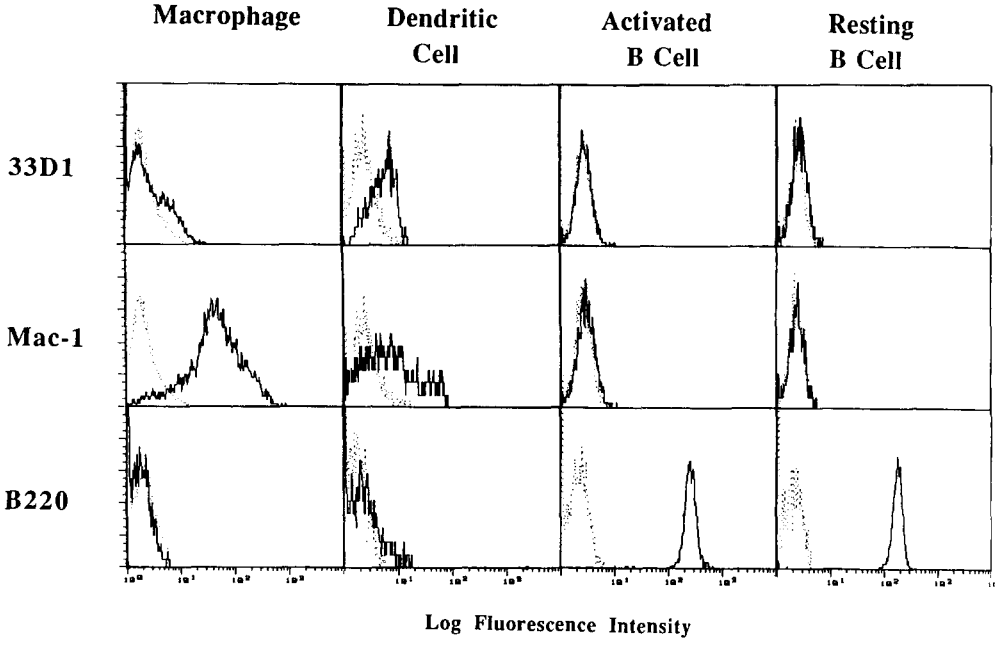

Figure 2. Antigen-presenting populations. APC were separated as described in Materials and Methods. Purity was assessed by staining with unlabeled 33D1 (lymphoid dendritic cell marker) and Mac-1 (macrophage marker) followed by FITC-RG7, and PE-conjugated B220 (B cell marker). Isotype-matched controls are shown with dotted lines.

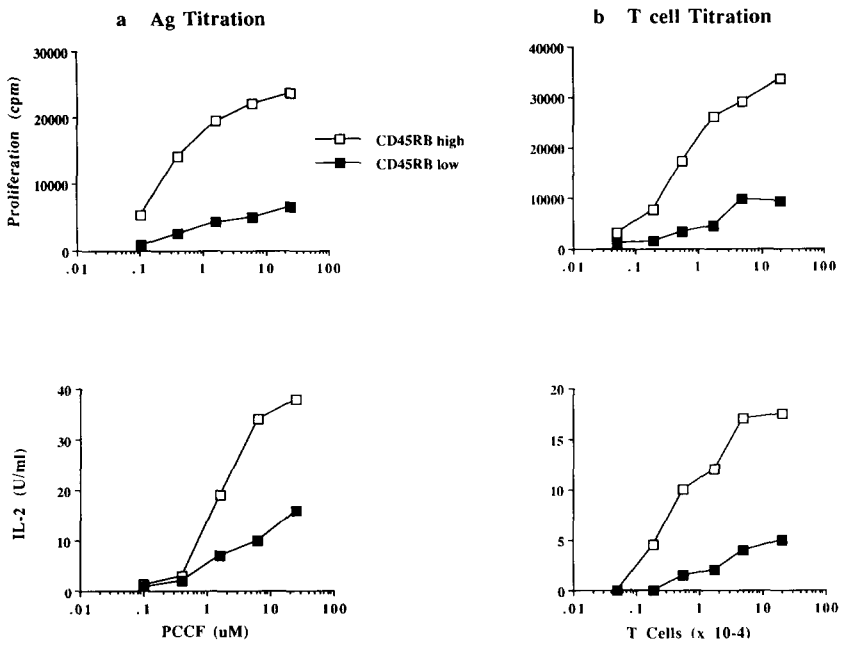

Figure 3. T cell proliferation and IL-2 secretion varies with antigen concentration and T cell number. (*a*) 2.5 × 10^4 T cells were added to an equal number of mitomycin-treated T-depleted splenic APC in the presence of varying amounts of PCCF peptide. T cell proliferation was measured between 48 and 72 h, and IL-2 secretion between 24 and 36 h. (*b*) 5 × 10^4 T-depleted APC were cultured with varying numbers of T cells and 10 μM PCCF. T cell proliferation and IL-2 secretion were measured as above. Results are from a representative experiment out of several performed, and show means of triplicate cultures. SEs (not shown) were <15% of the means.

erythrocytes and allogeneic cells have been widely studied, the ability of naive T cells to respond to soluble proteins has previously not been demonstrated in vitro. We found that CD45RB low cells from transgenic mice respond well to polyclonal stimulation by immobilized anti-CD3 (without exogenous APC), and that under those conditions they proliferate better and secrete more IL-2 than CD45RB high cells (data not shown). In the presence of exogenous APC, CD45RB low and CD45RB high cells responded equally to anti-CD3, and again with similar kinetics, suggesting that naive T cell response can be equivalent to memory T cell response if appropriate activation signals are given.

In separate experiments, we also directly analyzed cells derived from cultures of CD45RB high CD4 cells responding to PCCF and APC as above. As judged by cell counting and fluorescence analysis, 50% of the starting CD4 population had entered G_{1A} phase of the cell cycle by 24 h and exhibited increased forward scatter and increased levels of CD44 (Pgp-1), while retaining CD45RB. At 48 h after initiation of culture, 90% of CD4 cells present were large blasted cells that displayed uniformly increased levels of CD44. This suggests that a large proportion of the naive CD4 T cell population responds to antigen in these in vitro cultures.

Dendritic Cells Are the Optimum APC for Naive CD4⁺ T Cells. To investigate which APC were the best stimulators of naive T cells, we cultured CD45RB high transgenic cells with varying numbers of different mitomycin-treated APC in the presence of 10 μM PCCF. The peptide fragment was used so that differences in processing among the different APC would not influence the results. Because BMDM secrete high levels of prostaglandins, which are known to downregulate T cell responses, we added 5 μM indomethacin to all cultures. A representative experiment is shown in Fig. 4 (T cell proliferation at 48–72 h) and Fig. 5 (IL-2 secretion). Analysis of proliferation at earlier or later time points gave similar results (data not shown). Proliferation and IL-2 secretion in the absence of exogenous APC or Ag were negligible.

All APC populations induced T cell proliferation to a certain extent, but dendritic cells were better stimulators at low cell numbers, initiating a detectable response when <10^3 were added per culture (>1:25 APC/T ratio) and an optimum response at 10^4 per culture. Splenic macrophages and activated B cells were also very active, but responses were detectable only when 5 × 10^3 or more cells were present. Op-

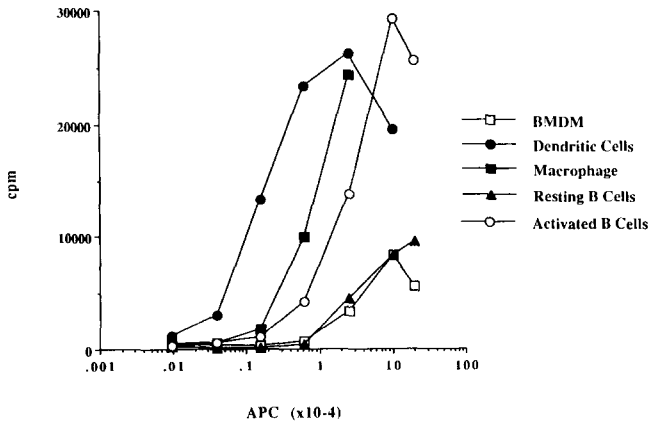

Figure 4. Dendritic cells are the most efficient stimulators of naive T cell proliferation. Varying numbers of purified mitomycin-treated APC were added to 2.5 × 10^4 CD4⁺, CD45RB high cells in the presence of 10 μM PCCF. T cell proliferation was measured at 48–72 h. Results show a representative experiment. Means are from duplicate cultures, and SDs were <15% of the means.

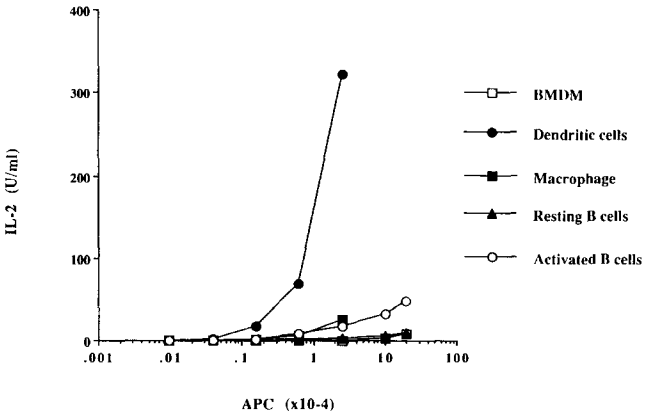

Figure 5. Dendritic cells are the most potent stimulators of naive T cell IL-2 production. 2.5 × 10^4 $CD4^+$, CD45RB high cells were added to varying numbers of purified mitomycin-treated APC and 10 μM antigen. IL-2 secretion was assessed after 24–36 h. A representative experiment is shown using duplicate cultures, with SDs being <15% of means.

timum responses for activated B cells required 5 × 10^4 cells. Resting B cells and BMDM were very poor stimulators of proliferation and only high numbers of these APC induced detectable responses.

Fig. 5 shows IL-2 production in similar cultures. Again, all APC populations induced detectable IL-2, although with BMDM and resting B cells at highest cell numbers, <10 U/ml were seen (the level of detection in this assay is 0.5 U/ml). Activated B cells and the splenic macrophage preparations induced significant amounts of IL-2 (50–100 U/ml maximum) at high APC/T ratios. In contrast, dendritic cells not only induced significant IL-2 at low cell numbers (17 U/ml with 1.5 × 10^3 cells), but induced massive IL-2 secretion at high concentrations. With the highest number of dendritic cells tested (10^5 cells), IL-2 levels in excess of 10^3 U/ml were obtained (data point not shown in Fig. 5).

Dendritic cells were therefore by far the most efficient APC for stimulating naive CD4 T cells with peptide antigen. Previous studies by Lassila et al. (16) had suggested that small B cells are unable to activate naive T cells, and in our experiments resting B cells also had little stimulatory activity. Large, activated B cells induced significant levels of proliferation and IL-2 secretion, although of much lower magnitude than dendritic cells. These cells were extensively purified and appeared to be dendritic cell free, supporting the concept that activated B cells can indeed stimulate naive T cells. Significantly, many B cell lines, where contamination with dendritic cells can be ruled out, are good stimulators of the MLR (17). The splenic macrophage population was contaminated with appreciable numbers of dendritic cells (Fig. 2), and considering the weak activity of BMDM, it is likely that much of the activity of macrophages may actually be attributable to contaminating dendritic cells. In other experiments, we have shown that a fibroblast cell line transfected with IE^k is an excellent APC for stimulating the naive CD4 T cells from transgenic mice (S. L. Swain and M. Croft, unpublished results). Thus, it appears that dendritic cells are the most efficient, but not the only APC, which can stimulate naive CD4 T cells.

There may be multiple features that make dendritic cells such efficient stimulators of naive $CD4^+$ cells. Dendritic cells express high levels of many surface antigens, including MHC class II, ICAM-1, LFA-1, LFA-3, and CD29 (15), which no doubt contribute to their ability to interact with T cells. The large surface area of the dendritic cells, coupled with the expression of coreceptors, probably allows presentation of antigen to several T cells at once, a feature not thought to occur with other APC (18). Furthermore, the avidity of the T cell–dendritic cell interaction may be stronger than with other APC, a phenomenon that is possibly critical for initiation of responses, as proposed by Sprent and Schaefer (19). In addition, dendritic cells may provide a second signal or costimulus (20). Recent evidence has shown that the molecule termed B7/BB1, present on many APC types, may well provide this costimulatory signal (21) by ligating its counterreceptors CD28 (22) and CTLA-4 (23) on the T cell surface. Resting, but not low density, B cells lack costimulatory activity, as demonstrated by Jenkins et al. (24), whereas it is possible that dendritic cells may constitutively be costimulatory by expressing B7/BB1.

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Address correspondence to Michael Croft, Department of Biology and the Cancer Center, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0063.

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