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Induction of CD4 T Cell Changes in Murine AIDS Is Dependent on Costimulation and Involves a Dysregulation of Homeostasis

Michael H. Yen, Nancy Lepak, and Susan L. Swain

Strong CD4 T cell activation and proliferation are seen in susceptible mice infected with the murine retroviral inoculum, LP-BM5, which produces an immunodeficiency syndrome called murine AIDS (MAIDS). We developed a short term adoptive transfer model of MAIDS to examine the requirements for the CD4 T cell response. Naive CD4 T cells from uninfected donors responded quickly after adoptive transfer into MAIDS-infected hosts, becoming activated and proliferating within several days. Using blocking mAbs to costimulatory ligands and CD4 T cells deficient in expression of their receptors, we found that the CD4 T cell response requires CD28:B7.1/B7.2 interactions, but not CTLA4 or CD40-CD40 ligand interactions. Naive CD4 T cells did not respond in H-2M-deficient mice with MAIDS, suggesting that disease requires recognition of self peptide-MHC complexes. The self MHC-dependent division and accumulation of large numbers of CD4 T cells suggest that MAIDS involves a disruption of the balance of homeostatic signals. Supporting this hypothesis, CD4 T cells from mice with MAIDS failed to regulate the homeostatic division of naive CD4 T cells in a cotransfer model. Thus, a combination of up-regulation of costimulatory ligands and disruption of homeostatic control may be responsible for CD4 lymphoproliferation in MAIDS. The Journal of Immunology, 2002, 169: 722–731.

Optimal stimulation of naive CD4 T cells is a complex process requiring the interaction of several different molecules on the surface of the T cells with molecules on the APC (1–3). Foremost is the ligation of the TCR by its appropriate Ag in context of MHC class II molecules, an interaction that is facilitated by CD4 coreceptor binding (4). Costimulation involves a second set of interactions between the T cell and the APC that provide greater stabilization and additional signaling to the T cell (1, 2, 5). The best characterized costimulatory pathway involves interaction of CD28 on the CD4 T cell and B7.1 or B7.2 (CD80/CD86) on the APC. A variety of other costimulation pathways have also been described (2, 3).

Efficient stimulation of naive CD4 T cells in vitro results in their proliferation, expansion, and development into an effector population (6). Expansion of naive T cells during primary response can be substantial, ranging from 50-fold for keyhole limpet hemocyanin (7) to 1200-fold for pigeon cytochrome c (8). Expansion of virus-specific CD4 T cells in C57BL/6 mice during the acute phase of lymphocytic choriomeningitis virus infection was recently reported to be >100-fold (9). The numbers of resting T cells are tightly regulated by ill-defined homeostatic mechanisms (10), and in conventional immune responses the expansion of CD4 T cells is transient, with effector cells quickly returning to baseline levels. A role for Ag-independent, cytokine-mediated proliferation of naive and memory CD4 T cells has also been proposed, but in general seems restricted to memory cells and does not increase the size of the homeostatically regulated T cell compartment (11–13).

Infection with the BM5 retrovirus induces a massive, polyclonal CD4 T cell response that results in accumulation of large numbers of CD4 T cells with activated/memory phenotype but that appear anergic rather than like active effectors (14). The mechanism by which the etiologic agent of disease, a replication-defective C-type murine leukemia virus called BM5d (15), causes disease is unknown. B cells are the major target of infection (16, 17), and they become activated and expand greatly in murine AIDS (MAIDS) (18, 19). Generation of disease is dependent on both B cells and CD4 T cells, and class II and CD4 expression is required, leading to the suggestion that MAIDS is an Ag-driven disease (20). However, there is no Vβ selectivity to the response, which seems to involve all T cells and can also occur in TCR-Tg (Tg) model (19, 21). This argues that Ag or superantigen recognition is not involved. In CD4-deficient mice, the B cells do not undergo disease-associated changes, supporting a role for CD4 T cell-B cell interactions in the B cell response of MAIDS (19).

Administration of blocking mAb to various costimulatory molecules and use of mice deficient in costimulatory receptors or ligands in whole animals infected with BM5 have suggested a role in MAIDS pathogenesis for B7.1 and/or B7.2 (22), ICAM-1, and LFA-1 (23), and CD40 ligand (CD40L) (24, 25). The precise mechanisms by which blocking or removing the molecules abrogate disease is unclear. Blocking mAb could act at any stage and by one or more of several mechanisms, including but not limited to 1) limiting spread of the virus, 2) modulating expression of costimulatory molecules on APC, 3) ablating direct effects of viral products on T or B cells, or 4) blocking other indirect effects due to viral infection and MAIDS pathogenesis, seems to be a multistep process (19, 26).

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3 Abbreviations used in this paper: MAIDS, murine AIDS; PCCF, pigeon cytochrome c fragment; Tg, transgenic; Green BODIPY, CellTracker Green BODIPY; CD40L, CD40 ligand; WT, wild type; HDD, homeostasis-driven division.
We have designed an adoptive transfer approach to precisely determine which molecular interactions are involved in the changes in CD4 T cells in MAIDS. We transfer resting naive donor CD4 T cells into normal hosts or infected hosts with advanced disease. Donor cells from AND TCR-Tg mice are Thy.1.2+ and express a Vβ3/Vα11 TCR that is specific for peptide 88–104 of pigeon cytochrome c (PCCF) presented in I-Ek (27, 28). After transfer, organs are harvested to analyze the donor cells, and donor CD4 T cells can also be recovered by resolation and tested for function. Donor cells are labeled with vital fluorescent dyes such as CFSE or CellTracker Green (Green BODIPY; Molecular Probes, Eugene, OR), which permits visualization of five or six divisions of cells (29, 30).

Our results indicate that donor CD4 T cells respond within a few days in the MAIDS environment, undergoing activation and proliferation. This response is blocked by concurrent in vivo treatment with blocking mAb to B7.1 and B7.2, but not by treatment with blocking mAb to CTLA4 or CD40L. In concordance, CD4 T cells deficient in expression of CD28 do not respond in BM5-infected hosts, whereas CD4 T cells deficient in expression of CD40L undergo MAIDS-related changes. Little CD4 T cell response occurs in H-2M-deficient mice with MAIDS, suggesting that their response occurs by a novel mechanism involving CD28 and B7 family interactions and recognition of self peptide class II complexes by the CD4 T cells.

Materials and Methods

Mice

B6.PL-Thy1.1/Cy mice (B6.Thy1.1) were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in our Animal Breeding Facility at the Trudeau Institute (Saranac Lake, NY). H-2d/b Vβ3/Vα11 and AND TCR-Tg mice, which are specific for PCCFa8–104 in MHC class II Ek (27), were bred in our facility and used at 2–3 mo of age. The AND mice were originally on a C57Bl/6 (B6 × SJL) background but were backcrossed >10 times to C57Bl/6. CD28–/– mice backcrossed 10 times to C57Bl/6 were kindly provided by Dr. T. Mak (Agening Institute, Toronto, Ontario, Canada), and used at 2–4 mo of age. C57Bl/6 bred in our facility were used as CD28–/– controls. (B6 × 129F2)F1, CD40L–/– mice were obtained from The Jackson Laboratory and bred in our facility; (B6 × 129F2)F1 mice were used as CD40L–/– controls. DC40 L–/– mice on the B6 × 129 background were originally obtained from Dr. R. Geha and bred in our facility, and B6 × 129 mice were used as CD40 L–/– controls. Mice used for mAb ascites production include C57Bl/6-nu/nu, CB.17/SCID, and BALB/cBy × DBA/2.

Virus

LP-BM5 (BM5) viral stocks were obtained as cell-free supernatants of chronically infected SC-1 cells (gift of Dr. M. Haas, University of California, San Diego, CA). Adult B6.Thy1.1 mice (5–8 wk of age) were infected i.p. with 0.5 ml of BM5 viral stock and compared with age-matched, sex-matched uninfected control mice. BM5-infected B6.Thy1.1 mice were used as hosts in the adoptive transfer experiments at 4 wk postinfection.

CD4 T cell preparations

Isolation of CD4 T cells has been described previously (28). Briefly, spleen and lymph nodes of donor mice were pooled, passed over nylon wool, and treated with depleting Abs to CD8a (3.155; American Type Culture Collection [ATCC], Manassas, VA), heat-stable Ag (J11d; ATCC), and class II (D3.137, C4A-A12.2, M5/114; ATCC) followed by mouse anti-rat x chain MARR18.5 (ATCC) and complement lysis. Small resting CD4 T cells were harvested from the bottom interface layer of a discontinuous Percoll gradient (four layers: 45, 53, 62, and 80; Sigma-Aldrich, St. Louis, MO). Remaining cells were 80–90% CD4, 90–95% of which displayed a naive phenotype (CD44hi, CD45RBlow, CD69lo), CD4 T cells from AND TCR-Tg mice expressed the Tg Vβ3/Vα11 TCR as identified by FACS staining.

Green BODIPY labeling

Purified small resting CD4 T cells were labeled with the vital dye Green BODIPY. This intracellular dye binds to thiol residues and fluoresces in channel 1 in FACS analysis. Cell were resuspended at 107 per ml in RPMI 1640 (Life Technologies, Gaithersburg, MD), and 5 μM Green BODIPY dissolved in DMSO (Sigma-Aldrich) was added to a final concentration of 5 μM. Cells were incubated at 37°C for 12–15 min. The reaction was stopped by addition of cold RPMI, and the cells were washed once and enumerated. Immediate toxicity was minimal in that 90–100% of the cells were recovered and were live as determined by trypan blue exclusion. Green BODIPY-labeled CD4 T cells responded as well as unlabeled CD4 T cells in vitro stimulation with plate-bound anti-CD3 (2C11) and anti-CD28 (37.51; gift of Dr. J. Allison, University of California, Berkeley, CA), as determined by [3H]Tdr incorporation.

Blocking mAbs

For blocking studies, the following mAb were used: anti-CD80 (B7.1; 16-10A1, ATCC); anti-CD86 (B7.2; GL1; ATCC); anti-CD152 (CTLA4; UC10-4F10); anti-CD154 (CD40L; MR1, gift of Dr. R. Noelle, Dartmouth Medical College, Lebanon, NH). Control rat IgG2a mAb LTF-3.6 (anti-keyhole limpet hemocyanin) was kindly provided by Dr. L. Johnson (Trudeau Institute). Control purified hamster IgG was purchased from Cappel Organon Teknika (Durham, NC). All mAb were purified by protein G affinity chromatography (Pharmacia Fine Chemicals, Piscataway, NJ) from hybridoma culture supernatants or ascites in pristane-primed nude, SCID, or irradiated and cortisone-treated (BALB/cBy × DBA/2)F1 mice. All mAb were passed over endotoxin-removing gel columns (Pierce, Rockford, IL) and sterile-filtered. Ab concentrations were determined by isotype ELISA. Some mAb were further concentrated using Centriplus YM-100 concentrators (Amicon, Beverly, MA).

Adoptive transfer protocol

For experiments involving blocking mAb, uninfected and BM5-infected B6.Thy1.1 host mice received i.p. 0.5 mg blocking mAb or isotype control or saline 6 h before the adoptive transfer of 5–20 million Green BODIPY-labeled donor AND CD4 T cells (day 0), followed by 0.5 mg mAb daily for 3 days. Hosts were sacrificed on day 4. For experiments involving knockout donor CD4 T cells (CD28–/–, CD40L–/–, CD40 L–/–), uninfected and BM5-infected B6.Thy1.1 host mice received 5–10 million Green BODIPY-labeled donor CD4 T cells from either the knockout mice or the appropriate control strain. Hosts were sacrificed on day 7.

Recovery of donor cells

At the conclusion of each experiment, uninfected and BM5-infected hosts were sacrificed, and spleen index measurements were taken as a rough indication of extent of disease. Spleen index was calculated by dividing the spleen weight-body weight ratio of the MAIDS-infected mice by the spleen weight-body weight ratio of the uninfected mice (31). Single-cell suspensions were made from individual mice from the spleen and lymph nodes and filtered through a 70-mm pore size nylon cell strainer (BD Labware, Franklin Lake, NJ), and the lymphocytes were counted. Samples of the unseparated cell suspensions were set aside for staining and FACS. Donor CD4 T cells were enriched from host cells by treatment with depletion Abs to CD8 (3.155, class II (D3.137, C4A), and Thy.1.1 (19E12; gift of Dr. R. Nowinski, Fred Hutchinson Cancer Research Center, Seattle, WA), followed by complement lysis. Donor cell frequency in the enriched population was determined by FACS, either by Vβ3/Vα11 staining or by CD4+ and Thy1.2+ staining.

Ex vivo function of donor AND T cells

Ex vivo function of adoptively transferred donor AND T cells was determined by proliferation and cytokine production in response to specific Ag PCCF presented by the DCEK. ICAM cell line. The DCEK. ICAM line has been previously described (3, 32). For Ag presentation, DCEK. ICAM was preincubated in 10 mM PCCF at 5 × 104 cells/ml at 37°C for 2 h, followed by extensive washing. Mitomycin C (100 mg/ml, Sigma-Aldrich) was added during the last 30 min. Donor AND T cells were plated at 4 × 104, 2 × 104, and 1 × 104 cells/well with 1 × 105 preincubated, mitomycin C-treated DCEK. ICAM, in 200-ml cultures in 96-well plates (Costar, Cambridge, MA). Cells were cultured in RPMI 1640 supplemented with penicillin, streptomycin, glutamine, 2-ME, HEPES, and 5.7% FBS (Intergen, Purchase, NY). Proliferation was measured at 60–84 h of culture by incorporation of [3H]Tdr (0.2 μCi/well) during the last 12 h.

Flow cytometry

Cell staining and analysis was performed on the unseparated cell suspensions from individual host mice after the adoptive transfer experiment. A panel of mAb-recognizing murine cell surface markers was added at appropriate concentrations to aliquots of 106 cells. Nonspecific binding of staining mAb was blocked by preincubation of cells on ice for 20 min with...
anti-FcγRIII mAb 2.4G2 (ATCC) and normal rat serum (Pel-Freeze Biologicals, Rogers, AR). Staining reagents include biotin anti-Thy1.2 (clone 30-H12, ATCC), PE anti-Thy1.2 (clone 30-H12; BD Pharmingen, San Diego, CA), biotin anti-CD4 (clone GK1.5; ATCC), PE anti-CD4 (clone RM4-5; Caltag Laboratories, Burlingame, CA), biotin anti-Vβ3 (clone KJ25, gift of Dr. J. Kaye, The Scripps Research Institute, La Jolla, CA), PE anti-Vα1 (clone RR8-1; BD Pharmingen), PE anti-CD44 (clone IM7; BD Pharmingen), PE anti-CD62L (clone MEL-14; BD Pharmingen), PE anti-CD5SRB (clone 23G2; BD Pharmingen), PE anti-CD69 (clone H1.2F3; BD Pharmingen), PE anti-CD25 (clone PC61; BD Pharmingen), PE anti-CD122 (clone TM-b1; BD Pharmingen), CyChrome anti-CD8α (clone 53-6.7; BD Pharmingen), and streptavidin-conjugated Cy5 (Caltag Laboratories). After staining with the appropriate staining reagents, cells were washed and resuspended in PBS containing propidium iodide at 0.2 μg/ml, and FACS was conducted immediately on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences). All plots shown are gated on the live lymphocyte population.

**Results**

**Kinetics analysis of in vitro responses to specific Ag**

In contrast to the more widely used CFSE, BODIPY dyes label thiois and in initial comparisons gave somewhat clearer results in vivo. For consistency, most remaining studies were conducted with BODIPY, although others with CFSE gave similar results. To provide a benchmark for using the BODIPY dye, we examined the in vitro proliferation of the naive CD4 T cells to be used in the study to Ag-pulsed APC. CD4 T cells isolated from AND TCR Tg mice were labeled with Green BODIPY and either stimulated in vitro with PCCF presented by DCEK.ICAM (3), or cultured in the absence of stimulation. Fig. 1a demonstrates that Ag-stimulated AND CD4 T cells proliferated from days 1 through 4 of culture, as determined by increasing recovery of CD4 cells. Unstimulated cells die in culture. Fig. 1b shows the Green BODIPY profiles taken on each day of culture and illustrates the rapid shift downward in BODIPY dye content when the cells are optimally stimulated. In contrast, unstimulated, nondividing cells exhibit only a slow dye loss which is probably due to protein turnover and/or leakage of the dye. By 4 days, in vitro stimulated cells become effectors that rapidly secrete high levels of IL-2 and other cytokines on restimulation (not shown).

**Adoptive transfers of dye-labeled CD4 T cells into normal mice or mice with MAIDS**

To evaluate the effects of BM5 infection in vivo, naive CD4 T cells from AND TCR-Tg mice were adoptively transferred into uninfected and BM5-infected B6.Thy1.1 hosts. After adoptive transfer, the donor cells could be clearly identified in the hosts on the basis of expression of the Tg TCR Vβ3Vα11 (Fig. 2), which is a rare event in normal or BM5-infected mice, and they can also be visualized by Thy1.2 expression (data not shown). Substantial differences are seen in the CD4 T cell fates in the uninfected and infected hosts. In uninfected hosts, donor cells are found only in small numbers (Fig. 2). Between days 1 and 4 after transfer, recoveries of donor cells from normal hosts range from 10 to 25% of the starting number of donor cells that were injected (Fig. 2b, □), a range of recovery of the transferred population is standard in this and other models of naive CD4 T cell transfer to normal hosts (33). In contrast, by day 4, donor cells recovered from infected hosts had undergone expansion (Fig. 2), reaching 150–300% of the starting number, a 15– to 30-fold increase relative to initial numbers in the spleen (~10% of those injected). Similar expansions were noted in other secondary lymphoid sites (not shown).

To further analyze the extent of the response of the naive CD4 T cells in MAIDS-infected hosts, we examined the BODIPY profiles and phenotypes of donor cells after transfer. Strong evidence that the expansion is due to multiple rounds of division can be seen in Fig. 3a, which shows loss of BODIPY dye content over a range of at least four divisions when cells were recovered from infected hosts but not from uninfected hosts. In other studies with 5-bromo-2′-deoxyuridine pulsing, we have found that the same naive CD4 cells transferred to normal hosts do not undergo division over a 1–2-wk period (not shown) but that naive cells slowly but progressively lose the dye, presumably due to degradation of short-lived proteins.

Donor cells in uninfected hosts remain small throughout the 4 days of analysis (Fig. 3b, light lines), and they maintain resting levels of CD44 (Fig. 3c, light lines) and other activation markers such as CD62L, CD45RB, CD69, and CD122 (data not shown). In contrast, donor cells introduced into infected hosts exhibit an increase in forward scatter, an indication of increased size (Fig. 3b, dark lines), and up-regulate CD44 (Fig. 3d, dark lines) as well as CD69, and they down-regulate CD62L (not shown). Levels of TCR and CD28 remain constant (not shown).

Extensive earlier studies in the MAIDS model indicate the CD4 T cells become profoundly anergic so that after ex vivo restimulation they do not divide, expand, or make cytokines nor do they respond to cytokines but instead die (34). Moreover, addition of IL-2, IL-12, and/or costimulation was unable to rescue the anergic population from mice with MAIDS (35). To determine whether the CD4 T cells in infected hosts show signs of progressing to such a state, we reisolated cells from MAIDS-infected hosts after 4 days. Donor cells from infected hosts mounted a weaker proliferative response to specific Ag (Fig. 3d, □) than donor cells recovered from uninfected hosts (Fig. 3d, □). The recovered cells did not divide in response to IL-2 (not shown). Thus, the CD4 T cells transferred to mice with MAIDS show proliferative anergy in only 4 days, and we have noted that it repeated after 7 days as well. This anergy is seen in CD4 T cells in the mice with MAIDS only after several weeks (14). Other manifestations of anergy have not been extensively studied here, because the small numbers of cells recovered make those analyses very difficult. Thus, we cannot conclude whether the anergy seen here in just a few days is as pro-

**FIGURE 1.** In vitro characterization of CD4 T cell expansion and loss of Green BODIPY dye content. a, Kinetics of in vitro CD4 T cell expansion. Naive CD4 T cells from AND TCR-Tg mice were purified and labeled with Green BODIPY vital dye, then cultured in vitro for 4 days, either in the absence of stimulation (□) or in the presence of specific Ag, PCCF, presented by I-Eκ (■). Live CD4 T cell recoveries were determined each day by trypan blue exclusion under light microscopy. b, In vitro expansion correlates with loss of vital dye content. On each day of culture, FACS was performed on the Vβ3′Vα11′ population to assess dye content. Data are representative of three experiments.
found or irreversible as that accomplished over the longer time in infection.

**Blocking B7-CD28 interactions prevents donor CD4 T cell responses in MAIDS-infected hosts**

A major advantage of the adoptive transfer model to analyze mechanisms of CD4 T cell changes caused by MAIDS is that it allows for short term mAb blocking experiments to investigate the requirement for various molecular interactions. Because the disease is already established and the donor cells respond immediately in the hosts, any effects of the blocking can be directly attributed to interference with CD4 T cell response. We treated the host mice during the adoptive transfer period with blocking mAb to candidate costimulatory molecules.

**FIGURE 2.** Donor CD4 T cells expand after transfer to hosts infected with BM5 (MAIDS). Naive CD4 T cells from AND TCR Tg mice were purified and labeled with Green BODIPY vital dye and 2 × 10^5 labeled cells were transferred into normal (◼) or hosts infected with BM5 4 wk previously (■). After 1–4 days, spleens and lymph nodes of recipients were harvested. Donor CD4 T cell recoveries were determined by FACS analysis for expression of Vβ3+Vα11+ by Thy1.2+ cells in each mouse. a, Visualization of donor AND Tg CD4 T cells in adoptive hosts. Analysis of cells recovered after 4 days in vivo. The number in the upper right quadrant of each dot plot is the percent of Vβ3+Vα11+ cells. b, Expansion of naive CD4 T cells in hosts with MAIDS. Donor CD4 T cell recoveries were determined by FACS of transgene-bearing Thy1.2+ cells. Bars, mean ± SE of groups of three to five individual mice.

**FIGURE 3.** Phenotype and ex vivo function of donor CD4 T cells recovered from hosts with MAIDS. Donor TCR-Tg naive CD4 T cells were labeled and transferred as in Fig. 2 except hosts were Thy1.1. From 1 to 4 days after transfer, recipient spleen and lymph nodes were harvested and enumerated with Abs to Thy1.2. Expression of CD44, CD62L, and CD45RB on Thy1.2+ gated donor cells was determined by FACS (a–c). Data are representative of three experiments. a, BODIPY dye loss. Green fluorescence was measured to reflect the BODIPY dye label. Dark lines indicate donor cells recovered from hosts with MAIDS; light lines are those from uninfected hosts. b, Forward scatter (FSC). Dark lines indicate donor cells recovered from hosts with MAIDS; light lines are those from uninfected hosts. c, CD44 expression on gated donor cells recovered each day. Dark lines indicate donor cells recovered from hosts with MAIDS; light lines are those from uninfected hosts. d, Anergy in recovered donor cells. Donor cells were enriched from recipient spleen and lymph node. The donor cells were titrated at various cell concentrations corrected for donor cell frequency as determined by FACS and were stimulated in vitro with mitomycin C-treated APC pulsed with specific Ag, PCCF. Division was assessed at 72 h by pulsing with [3H]TdR for the last 12 h. Mean incorporation of radiolabel in replicate cultures ± SE are shown. ◼, Normal hosts; ■, MAIDS-infected hosts.
We initially investigated the effects of B7.1 and B7.2. Doses of specific Ab were chosen that block the response of naive TCR Tg cells in vivo. Groups of control, infected animals were injected with irrelevant Ab of the same isotype (isotype control). Transferred donor CD4 T cells did not expand in uninfected mice treated with either isotype control or the combination of anti-B7.1 and B7.2 (Fig. 4a). Donor cells expanded markedly in MAIDS-infected hosts treated with the isotype control Ab. Addition of either B7.1 or B7.2 alone did little to prevent the expansion of the transferred naive CD4 donor T cells in MAIDS-infected hosts (Fig. 4a). However, when Ab to both B7.1 and B7.2 were introduced, donor CD4 T cell recovery from MAIDS-infected hosts was reduced to a range comparable with the recovery from normal hosts (Fig. 4a).

A similar pattern was seen for division as determined by loss of BODIPY dye (Fig. 4b) and on activation as indicated by increased CD44 expression (Fig. 4c). Blockade of both B7.1 and B7.2 largely prevented donor cell division in MAIDS-infected hosts, determined on day 4 (Fig. 4b, shaded histogram). Together the Ab also prevented up-regulation of CD44 expression (Fig. 4c, shaded histogram). The combination also blocked other indications of activation and response such as down-regulation of CD62L and CD45RB and up-regulation of CD122 (data not shown).

**Blocking CTLA4-CD28 and CD40L-CD40 interactions does not prevent donor CD4 T cell changes in MAIDS-infected hosts**

Because B7.1 and B7.2 can bind to CTLA4 as well as CD28 (36), we sought to determine which molecule was the relevant target. Donor CD4 T cells were transferred to uninfected (normal hosts) and MAIDS-infected hosts, and groups were treated with either isotype control Ab or Ab to CTLA4. Treatment with anti-CTLA4 did not block the donor CD4 T cell response in the MAIDS environment (Fig. 5). Donor cell recovery was 10-fold higher from MAIDS-infected hosts than from normal hosts, despite the anti-CTLA4 treatment (Fig. 5a, gray bar). Donor cell proliferation in the presence of anti-CTLA4 mAb was confirmed by loss of BODIPY dye content (Fig. 5b). Activation, assessed by up-regulation of CD44 (Fig. 5c), and modulation of other activation markers (data not shown) also occurred in the presence of anti-CTLA4.

To further analyze the molecules involved, we blocked CD40-CD40L interactions by using the nonstimulatory and nondepleting anti-CD40L mAb MR-1. Blocking CD40L did not significantly reduce expansion of donor CD4 T cells in MAIDS-infected hosts (Fig. 5a, diagonally striped bar), and BODIPY profiles on day 4 indicated that the donor cells proliferated extensively in infected hosts regardless of whether the hosts were treated with isotype control Ab or anti-CD40L mAb (Fig. 5b). The donor cells also up-regulated CD44 (Fig. 5c, bottom two panels) irrespective of CD40L blockade by MR-1.

**CD28, but not CD40L expression by donor CD4 T cells is required**

Earlier analyses noted a delayed development of MAIDS in knockout mice deficient in CD28 or CD40L. However, lack of disease development in such models could be due to inhibition at any point along the multistep pathogenesis of MAIDS. Moreover, it cannot be deduced from either the mAb blocking or the previous knockout studies which cells needed to express CD28. In the adoptive transfer, we can focus exclusively on the mechanism of response of

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**FIGURE 4.** Both B7.1 and B7.2 are required for the CD4 T cell changes in MAIDS. Donor CD4 AND cells were prepared and transferred, and hosts were analyzed as in Figs. 2 and 3. a, Donor cell recovery from normal and MAIDS-infected hosts treated with blocking mAb to B7.1 and B7.2. Hosts received 0.5 mg each of mAb i.p. at 6 h before adoptive transfer of the donor cells (day 0), then every 24 h for 3 days. Hosts were sacrificed at day 4, and recovery of donor cells was determined. Host, from left to right, included normal control B6 mice with either isotype control or the combination of anti-B7.1 and B7.2, or hosts with MAIDS and either isotype control, individual Ab, or the combination. Results are expressed as the percent of transferred donor cells recovered, to compensate for slightly different numbers of donor cells transferred to control and MAIDS hosts. Bars, mean ± SE of three to five host mice in each group and are representative of three separate experiments. b, Donor cell division. In the same experiment as above, the division of the donor CD4 T cells was assessed by determining the dye content of donor cell gated recovered CD4 T cells. Top panel, Thin lines represent the histogram of donor cells recovered from normal hosts, and thick lines are those from infected hosts. Second panel, The shaded histogram represents the donor cells recovered from infected hosts treated with both B7.1 and B7.2. Third and forth panels, Those from infected mice with either anti-B7.1 or B7.2 alone. c, Phenotypic conversion of donor CD4 T cells. Naive CD4 T cells transferred into normal hosts (thin histogram) and MAIDS hosts (thick histogram, top panel), with either B7.1 or B7.2 blocked (thick histograms, bottom two panels) or when both B7.1 and B7.2 are blocked (shaded histogram).
CD4 T cells by using donor cells derived from knockout mice, because the infected control hosts are intact, and therefore disease progression is unchanged. Because only the donor T cell expresses the defect, if there is a lack of response, the target is clearly the donor T cell.

Unlike the donor CD4 T cells used in the previous studies, the CD28−/− or CD40L−/− CD4 T cells in these experiments are not Tg for the AND TCR. With nontransgenic CD4 T cells in MAIDS-infected hosts, the CD4 T cell response seen is somewhat less pronounced (Fig. 6a) and is biphasic (Fig. 6b, third panel), with some cells becoming activated and dividing as quickly as the AND TCR Tg donor cells and others not dividing at all by this time point. Nevertheless, obvious differences can be seen when nontransgenic cells are placed in the MAIDS-infected hosts as compared with uninfected hosts (Fig. 6b).

When CD28-deficient donor cells were transferred to uninfected mice, they were recovered at day 4, in numbers comparable with those of wild-type (WT) donor cells (roughly 25% of the starting input number), indicating that, as expected, the lack of CD28 did not impact survival (Fig. 6a). The donor cell recoveries of nontransgenic WT CD4 T cells from MAIDS-infected hosts were increased compared with uninfected hosts, and this enhancement was not seen when the donor CD4 T cells were CD28 deficient. When donor CD4 T cells from WT or CD28−/− mice were transferred into normal hosts, analysis of vital dye content indicates that very little if any cell division had taken place (Fig. 6b). When WT CD4 T cells were transferred into MAIDS-infected hosts, there was a clear dye loss in a portion of the cells, confirming that at least a cohort of donor cells were actively proliferating (Fig. 6b). At this point (4 days), some of the donor cells have not divided, but
with more time, most if not all of the nontransgenic donor CD4 T cells in MAIDS-infected hosts will respond by becoming activated and dividing (data not shown). When CD28<sup>−/−</sup> CD4 T cells were transferred into MAIDS-infected hosts, few donor cells divided (Fig. 7b). In a recent experiment, CD28<sup>−/−</sup> CD4 T cells expressing the AND TCR transgene also failed to proliferate or show signs of activation after transfer (data not shown). These results indicate that CD28 on the CD4 T cells must interact with B7.1/ B7.2 on host APC for the proliferative response to occur. The CD28<sup>−/−</sup> donor CD4 cells also failed to up-regulate CD44 (not shown).

In contrast to the importance of CD28, CD40L did not need to be expressed by donor CD4 T cells for them to respond in a MAIDS-infected host. The increase of donor cells from MAIDS-infected hosts was comparable whether or not the donor cells expressed CD40L (Fig. 6c). Both WT and CD40L<sup>−/−</sup> donor cells exhibited similar bimodal BODIPY profiles (not shown), with the dividing population displaying an activated surface phenotype (data not shown). This was in concordance with the negative results of our anti-CD40L blocking mAb studies. Thus the requirement for CD40 reported in earlier whole mouse studies models must reflect a requirement at some other step in disease progression.

**MAIDS and homeostatic recognition of self peptide/class II**

Considerable evidence supports a role for T-B interaction in B cell proliferation, activation, and anergy in MAIDS (19), yet the CD4 T cell response is broadly polyclonal, involving essentially all CD4 T cells, and is seen as well with individual TCR Tg CD4 T cells, arguing that there is no classic Ag recognition involved (19, 21). Yet, other instances of CD4 T cell activation and proliferation are almost always dependent on TCR triggering. Recently, it has become clear that peripheral CD4 T cells do receive positive signals from recognition of self peptide/class II and that in circumstances where other T cells are not providing negative feedback, these signals can drive their division and expansion (37). To examine whether signals delivered by recognition of self class II/peptide are playing a role in CD4 expansion in MAIDS, we used H-2M<sup>−/−</sup> mice that are deficient in a key molecule involved in peptide loading (38, 39), so that they express a restricted spectrum of self peptides, and thus a limited CD4 T cell repertoire. This limited repertoire is nonetheless able to substantially fill a peripheral CD4 T cell compartment. We tested whether H-2M<sup>−/−</sup> mice infected with BM5 would develop the MAIDS syndrome. Infected H-2M<sup>−/−</sup> mice developed all indications of MAIDS by 6–7 wk including splenomegaly (Fig. 7a), CD4 T cell expansion and B cell activation (not shown). Symptoms were slightly delayed compared with those in WT hosts. Like normal infected hosts, the CD4 T cell population in H-2M<sup>−/−</sup> mice infected with BM5, became anergic as indicated by loss of ability to produce IL-2 ex vivo in response to anti-CD3 plus CD28 stimulation (Fig. 7b).

To examine whether CD4 T cells undergo the division and expansion associated with MAIDS when they cannot recognize host class II/peptide in the homeostatic sense, we transferred AND TCR Tg CD4 T cells (positively selected on unknown peptide(s) with I-A<sup>δ</sup>) into H-2M<sup>−/−</sup> mice with MAIDS. The TCR Tg CD4 T cells were harvested after 48 h, and the titer of IL-2 was determined.

![Figure 7](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)

**FIGURE 7.** CD4 T cell recognition of self peptide/class II is required for their response. H-2M<sup>−/−</sup> mice were infected with BM5, and the symptoms of MAIDS were evaluated after 6–8 wk. Other infected H-2M<sup>−/−</sup> mice were used as hosts for AND TCR Tg CD4 T cells. a, H-2M<sup>−/−</sup> mice develop splenomegaly after infection with BM5. Groups of five control and BM5-infected control B6 and H-2M<sup>−/−</sup> mice were analyzed 8 wk after infection. Splenomegaly was weighed, and the mean ratio of the infected weights to the uninfected weights is shown. b, Anergy in H-2M<sup>−/−</sup> mice with MAIDS. CD4 T cells were purified from the above groups of H-2M<sup>−/−</sup> mice, and they were stimulated in vitro with plate-bound anti-CD3 plus anti-CD28. Supernatants were harvested after 48 h, and the titer of IL-2 was determined. Uninfected; , infected. c, Donor AND Thy1.1 CD4 T cell division in H-2M<sup>−/−</sup> hosts with MAIDS. Donor TCR-Tg CD4 T cells were labeled with CFSE and transferred to uninfected and infected B6 or H-2M<sup>−/−</sup> hosts. After 7 days, the dye content of Thy1.1 donor cells was determined. Percentages refer to the percent undivided donor cells (M1 M1). Histograms are representative of those seen in six to eight mice from two experiments. d, Donor AND Thy1.1 CD4 T cell expansion in H-2M<sup>−/−</sup> hosts with MAIDS. Bars, absolute numbers of donor cells recovered from pooled spleens and lymph nodes, expressed as means ± SE errors of results from individual mice. Similar results were seen in a repeat experiment.
were CFSE labeled. It is unlikely that such T cells, originally selected on some particular self peptide(s), would be able to recognize the limited peptide array expressed in the H-2M<sup>−/−</sup> host. Indeed, the transferred donor cells divided extensively in B6 MAIDS-infected mice but did not divide or expand in the H-2M<sup>−/−</sup> hosts (Fig. 7c). Donor cells expanded in B6 hosts but failed to expand in the H-2M<sup>−/−</sup> hosts. Similar results were seen in two experiments in a total of eight H-2M<sup>−/−</sup> hosts, all which showed clear signs of MAIDS-induced splenomegaly and host CD4 T cell expansion. These results suggest that along with the costimulatory requirements, CD4 T cells do not undergo division and expansion in response to MAIDS unless they also can interact in a homeostatic manner with self peptide/class II.

In lymphopoenic or T-depleted mice, where naive CD4 T cells undergo homeostasis-driven division (HDD), introduction of T cells can suppress that division (40). Mice with MAIDS have large numbers of memory phenotype CD4 T cells, and these could potentially suppress HDD. It is possible, however, that the anergic state of the MAIDS CD4 T cells renders them unable to suppress HDD. To test this possibility, we determined the effect of cotransferring CD4 T cells from mice with MAIDS, and from two other CD4 memory phenotype populations with naive AND CD4 T cells into T-depleted mice (Fig. 8). When transferred alone (Fig. 8, left), the naive CD4 T cells expanded (Fig. 8, left), recoveries of 23 × 10<sup>5</sup> from spleen alone, instead of an expected 1 × 10<sup>5</sup> routinely recovered from spleens of intact mice over 7 days (S. L. Swain, H. Hu, and G. Huston, unpublished observations) for the 2 × 10<sup>5</sup> introduced. The donor cells also divided as shown by loss of CFSE and became CD44<sup>high</sup> (not shown). In the experiment in Fig. 8 (right), only 3-fold fewer naive CD4 T were transferred, and total naive recovery was proportionally decreased, but cells divided comparably and expressed higher levels of CD44 (not shown). When 2 × 10<sup>5</sup> rested effector cells (33) or cells isolated from IL-2R knockout mice that are enriched in memory phenotype cells were cotransfereed with the naive cells, recovery was strongly reduced (Fig. 7, left), and donor cells divided little and did not show a shift to high expression of CD44 (not shown). However, the cells from mice with MAIDS, which have a similar memory phenotype (14), had no impact on cotransferrred naive CD4 T cells, even though in this case 4 × 10<sup>7</sup> MAIDS CD4 T were cotransferrred with the smaller number of naive CD4 T cells. The naive cells expanded comparably (Fig. 8, right), lost CFSE, and up-regulated CD44 (not shown). Thus, the CD4 T cells from mice with MAIDS are unable to regulate normal naive CD4 T cells in a homeostatic manner.

**Discussion**

Using an adoptive transfer model, we have been able to identify molecules and mechanism involved in the response of CD4 T cells in the MAIDS syndrome. We find that CD4 T cells must express CD28 and be able to interact with cells expressing B7 molecules. Interactions of CTLA4 with B7 or CD40L with CD40 are not necessary. Interestingly, despite a lack of conventional Ag specificity, TCR interactions with self class II/peptide seem to be required. This dual requirement for self-recognition and costimulatory interactions suggests a novel model in which up-regulation of B7 on APC, and possibly other virus-induced changes, leads to an imbalance in the normal homeostasis of CD4 T cells, allowing their proliferation and accumulation in large numbers.

In the adoptive transfer model where naive CD4 T cells are placed in infected hosts, we find a pattern of changes in the donor cells within 4 days after transfer. This pattern mimics the major changes reported to occur for CD4 T cells in whole animal infection over several weeks (14) including activation, division, and expansion of the CD4 T cells (Fig. 2). The naive TCR-Tg CD4 donor cells rapidly modulate surface activation markers including CD44 and become blasts, expanding in number as they undergo multiple rounds of cell division. When the donor cells are recovered from infected mice after 4–7 days, the cells proliferate little in vitro in response to stimulation with specific Ag (Fig. 3), which is also a feature of the in situ response (14, 34, 35).

Using blocking mAb to costimulatory receptors, we found that CD28:B7 family interactions are critical for the CD4 T cell response. A combination of Ab to B7.1 and B7.2 effectively blocks activation, division, and expansion of donor T cells (Fig. 3). Blockade of both B7.1 and B7.2 was required to prevent donor CD4 T cell changes, suggesting that B7.1 and B7.2 play redundant roles in the induction of CD4 T cell changes in MAIDS. Importantly, CD28-deficient donor cells do not respond (Fig. 6), indicating that CD28 on the T cells must interact with B7.1 or B7.2, presumably on host cells, most likely the many activated B cells expressing these molecules (19). We earlier showed that BM5 infection not only causes B cell hyperproliferation and polyclonal Ig secretion but also leads to up-regulation of both B7.1 and B7.2, as well as class II on the B cells (19). Blocking with Ab to CTLA4 had no impact, confirming the unique CD28:B7 requirement.

An earlier study indicated that CD28:B7 interactions were important for full disease progression and pathogenesis after BM5 infection (22). These studies did not discriminate between B7.1 and B7.2, and did not directly rule out a role for CTLA4, which is also capable of interacting with B7.1 and B7.2. Moreover, because the stage and target of the effects was not determined, no mechanism could be inferred.

Ligation of CD28 by stimulatory mAbs increases IL-2 gene enhancer activity, stabilizes IL-2 message, and promotes cell survival, perhaps by up-regulating Bcl-x<sub>L</sub> (5). In MAIDS, however, CD4 T cells become anergic to ex vivo stimulation so that they do...
not proliferate or produce of IL-2 in vivo or ex vivo on restimulation. Even if additional costimulation through CD28 or exogenous IL-12 is provided in vitro, the cells do not respond. The requirement for CD28-B7 interaction suggests a heretofore unappreciated form of anergy induction that is not due to absence of costimulation but is in fact dependent on such costimulation. As implied by the name, CD28 engagement alone is not sufficient to trigger proliferative responses of CD4 T cells but only costimulates TCR-mediated induction.

Recently, it has become clear that naïve T cells (both CD4 and CD8) will divide at a modest rate in hosts with low levels of endogenous T cells (13, 29, 37). This division requires recognition of self MHC and self peptides but does not require CD28 (40, 41). The results suggest that the resting, nondividing state of naïve T cells is the result of an equilibrium between positive signals, from interactions with self MHC/peptide and survival signals and negative signals directly from, or indirectly due to, the presence of other T cells. When the negative signals are lost, division, conversion to a responding cell phenotype, and expansion of the CD4 population occur. Cells remain functional and have some, but not all, of the properties of Ag-experienced cells (42, 43). The CD4 T cell response during MAIDS is both similar to, and different from, this HDD. We show here that H-2M b/c mice develop MAIDS but that naïve AND TCR-Tg CD4 T cells transferred to H-2M b/c hosts with MAIDS neither divide nor expand (Fig. 8). Such hosts have a limited T cell repertoire selected on the limited array of peptides that can be expressed in the absence of the H-2M molecule (38, 39). The fact that endogenous CD4 T cells, which have been selected on the self peptides present, do respond by expansion and development of anergy after infection of the H-2M b/c with BM5 but that the AND TCR Tg mice, selected in normal mice on unknown peptides, do not strongly supports the concept that TCR-self peptide/self MHC recognition is required for initiation of the CD4 T cell response in MAIDS. Thus, we suggest recognition by TCR of self peptide(s)/MHC is a component required to drive the CD4 T cell response seen here. However, the response of transferred CD4 T cells in MAIDS differs from HDD in that it occurs in mice that have CD4 T cells in abundance, although they are largely anergic. The fact that CD4 T cells accumulate at numbers in great excess of normal (10-fold expansion or more (14)) suggests a major dysregulation of homeostasis. Such a dysregulation could theoretically occur for several reasons that depend on how homeostasis is maintained, which is not well understood. If homeostasis is maintained by negative signals from other T cells, those signals could be missing in MAIDS or not be produced by the T cells that have responded in infected host. If it is maintained by a limited supply of a niche or factors, that supply could be increased during MAIDS. Alternatively or in conjunction with the above, the up-regulation of costimulatory molecules could also alter balance of the normal equilibrium in favor of response. We find here that the expanded CD4 T cells in mice with MAIDS do not homeostatically regulate naïve CD4 T cells in a cotransfer model. This supports the hypothesis that normal T cells play an active role in suppressing HDD and that when cells become anergic due to MAIDS they lose their ability to regulate and thus foster an MHC-dependent division of CD4 T cells.

The fact that the CD28:B7 and self MHC/peptide pathways can synergize to drive the substantial CD4 T cell changes in MAIDS, just as they synergize in Ag-specific responses, may imply that these pathways may interact to drive other T cell lymphoproliferative disorders.

Disrupting CD40-CD40L interactions had no impact in the transfer model (Figs. 5 and 6), whereas earlier experiments involving blockade in whole animals or infection of CD40L−/− mice suggested that they were required during disease in whole animals. Thus, it is likely that CD40-CD40L interactions are required during the initiation of disease, perhaps via the up-regulation of costimulatory molecules, such as B7.1, B7.2, and ICAM-1 on the surface of the APC. Once the B cells are expressing these molecules, there may be no further requirement for CD40-CD40L interactions. This is consistent with the activation of B cells as a prerequisite of CD4 T cell changes, as we have previously suggested (19).

Two major mechanisms have been hypothesized to explain MAIDS. One theory recognizes the requirement for myristilation and an intact gag molecule for pathogenicity and suggests that an association via a putative SH3-binding domain in gag, possibly with c-abl, results in altered signal transduction in infected B cells. Our results suggest that B cell activation and expression of B7.1 and/or B7.2 are critical steps in the MAIDS syndrome; this could be an important component of the total mechanism. The second theory focuses on the requirement for CD4 T cells and for class II expression (though these have not been dissociated) and postulates a role for aberrant Ag presentation in the initial events. We suggest that CD4 T cells interacting with self peptide/MHC on APC expressing B7 respond by division without development of effector function. Our results further suggest that a key feature of disease is a disruption of homeostatic equilibrium, which normally keeps CD4 T cells in a resting state and prevents them from accumulating to abnormal levels occurs.

Because the hosts in these experiments already have disease, it can be argued that the model pertains not to the initiation of MAIDS but instead to a later stage. This is certainly possible, but it is also possible that the events do mimic what happens as new naïve CD4 T cells are exposed to APC in infected mice.

The adoptive transfer approach to studying MAIDS could easily be applied to other diseases where it would afford a novel opportunity to examine the CD4 T cell response in a complex multistep disease model. This approach may also prove useful in studying specific lymphocyte populations in other diseases, where it has often been impossible to focus on particular steps in the often dauntingly complex infectious process. Moreover, understanding aberrant CD4 T cell responses that lead to anergy may provide insights into the development of immunodeficiency in other circumstances, as well as into the mechanisms involved in homeostasis.

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References


