The functional and phenotypic heterogeneity of CD4+ T cells has recently evoked considerable attention. A major question raised in these investigations is whether such subsets are generated and maintained as distinct "lineages" or, instead, represent different stages of activation and maturation. While recent studies support the latter interpretation (1), it remains unclear how the subdivision of murine T helper lines into Th1 and Th2 on the basis of distinctive lymphokine secretion (2, 3) can be incorporated into such a scheme. Here, in an extension of our earlier work resolving murine CD4+ T cells into four subsets (4), we demonstrate that activation and maturation of T cells results in permanent alteration of surface phenotype and that such alteration is coupled with a change in the lymphokines secreted. That is, whereas cultures of primary T cells secrete IL-2 predominantly (without IL-4), cultures of mature memory T cells generated in response either to soluble antigen or to allogeneic cells secrete IL-4 predominantly (without IL-2). We further demonstrate that IL-4-secreting memory T cells show two distinctive stable phenotypes depending on the type of antigenic stimulation used in their induction.

Materials and Methods

Mice and Immunization. BALB/cAnN and C57BL/6JN mice were bred and maintained in our animal facility. BALB/c mice were immunized with Keyhole limpet hemocyanin (KLH) as described previously (4).

Immunofluorescence Analysis and Cell Sorting. Spleen cells from either unprimed or KLH-primed BALB/c mice were stained simultaneously with fluorescein (FL)-SM6C10, biotin-SM3G11 (revealed by phycoerythrin [PE]-avidin), and allophycocyanin (APC)-GK1.5 (anti-CD4), and analyzed or sorted using a dual-laser FACStar plus (Becton Dickinson & Co., Mountain View, CA) (4).

MLR. Unseparated spleen cells or CD4+ T cells from BALB/c mice were cultured with irradiated C57BL/6 spleen cells as described in Table I. Cell densities for the primary and secondary MLR are described in Table I and in the legend for Fig. 1. Growth factor (G.F.) was measured by the 3-(4,5-dimethylthiazol-z-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using the factor-dependent cell line HT2, where 50% of maximum growth was defined to be 1 U in 0.1 ml (2,4). Relative levels of IL-2 and IL-4 in the supernatant are determined
from percent inhibition of G.F. activity with the HT-2 cell line by anti-IL-2 (S4B6) or anti-IL-4 (1IB11) antibodies.

**Anti-KLH Antibody Response.** An in vitro secondary anti-KLH antibody response was obtained by incubation of fractionated CD4+ T cell subsets (1.5 x 10^5 cells/well) together with antigen (KLH)-pulsed B220+ (IgM, IgD)- cells (10^4 cells/well) as described previously (4, 5). Response is expressed as IgG1 anti-KLH secretion in 7–10-d supernatant as measured by an ELISA (4). 1 U anti-KLH corresponds to the IgG1 in a 1:10^5 dilution of BALB/c secondary anti-KLH antiserum.

**In Vivo "Parking" of Memory T Cells.** In vitro stimulated CD4+ cells were restimulated several times by appropriate stimulators to expand the cultures, and then injected intravenously (1–7 x 10^6 cells/mouse) into sublethally irradiated (130 Cs, 350 rad) C.B-17 scid mice ("parking"). CD4+ cells found in recipient scid mice were primarily transferred donor cells since they expressed CD3 (data not shown), in contrast with the general absence of CD3 from rare (0.3%) CD4+ cells in spleens of unmanipulated scid mice (A. Carroll, M. Bosma, K. Hayakawa, and R. Hardy, unpublished observation). Parked cells returned to the smaller size typical of CD4+ cells found in normal mice.

**Results and Discussion**

In a previous study (4), we showed that CD4+ cells in murine spleen are composed of four subsets that differ in their expression of cell surface determinants (3G11 and 6C10) recognized by two mAbs (Fig. 1, left). These phenotypically distinct subsets also differ functionally. That is, activation with the T cell mitogen Con A elicited IL-2 (without IL-4) from Fr.I and IL-4 (without IL-2) from Fr. III. To understand how these phenotypically and functionally distinct populations might arise in vivo, and how they are related, we initiated a study of the activation and maturation of CD4+ T cells with regard to changes in their phenotype and function. Two different types of in vitro stimulation were used to activate CD4+ T cells: (a) the response to allogeneic cells known as the MLR; and (b) the response to soluble antigen (KLH) in the induction of antibody secretion.

A primary MLR can be generated by coculturing CD4+ cells sorted from BALB/c spleen together with irradiated C57BL/6 spleen cells. Among the subsets of CD4+ cells, only cells from Fr. I and II were able to respond (Table I). FACS analysis of cultures initiated either with total CD4+ cells or with Fr. I or II showed an accumulation of large autofluorescent CD4+ cells of Fr. IV and III phenotypes, with Fr. IV finally predominating (Fig. 1 A, middle). The initial increase of Fr. III is probably due to a "bystander" (nonspecific) activation since only Fr. IV cells responded when rechallenged with appropriate stimulator cells (Fig. 1 A, middle). These cultures retained Fr. IV phenotype after repeated stimulation with irradiated allo-spleen cells.

An alternative system for investigating CD4+ memory T cells is the secondary humoral antibody response, which involves antigen presentation by memory B cells (5). As we reported previously (4), the CD4+ cells that help humoral antibody secretion after a primary in vivo immunization with KLH are localized in Fr. II and III and become restricted to Fr. III in older aged mice. Upon stimulation with antigen-pulsed memory B cells, Fr. II cells sorted from primed animals undergo a rapid loss of 3G11, generating cells with a Fr. III (and also some Fr. IV) phenotype (Fig. 1 B, middle). Most of the comparably stimulated, sorted Fr. III cells maintain their initial phenotype (Fig. 1 B, middle). These Fr. III cells can be repeatedly restimulated to proliferate with antigen-pulsed memory B cells and, although some Fr. IV cells do accumulate in these cultures, resorting experiments demonstrate that the
Phenotypic distinction between memory T cells for the allogeneic MLR and those that help anti-KLH antibody secretion. Primary allogeneic MLF (A) and secondary anti-KLH response (B) were used for activation and generation of CD4+ memory T cells. Four CD4+ T cell subsets were defined as Fr. I-IV according to their correlated cell surface expression of 3G11 and 6C10 as described previously (4). Fr. I and II (for allo-MLR) or Fr. II and III (for secondary anti-KLH response) were used to examine phenotypic alterations induced by activation (boxed with solid lines in the left panels). Middle panels show proliferating CD4+ cells 7-10 d after antigenic stimulation in culture. Right panels show CD4+ cells in recipient mice 12 d after transfer (11% in spleen A and 3% in spleen B) of memory T cell populations maintained in vitro. Boxes demarcating Fr. III and IV are the sorting gates for functional analysis to examine the distribution of memory T cells.

Majority of the helper activity remains in Fr. III (Fig. 1B, middle). Thus, our data demonstrate preferential expression of 6C10 (as Fr. III) by the memory T cells that help antibody secretion to soluble antigen (KLH) and an absence of 6C10 (as Fr. IV) from the in vitro memory T cells that respond in the allogeneic MLR.

To determine if resting CD4+ memory T cells induced by these two stimuli maintain these phenotypic distinctions, we injected the cultured cells into H-2 syngeneic C.B-17 scid mice, which provide a hospitable environment permitting a return to the resting stage ("parking;" reference 6). The results shown in Fig. 1, right, essentially confirmed the association of distinct functions with distinct phenotypes.

Remarkably, we found that these phenotypically distinct memory T cell populations established by successive in vitro antigen stimulation principally secrete IL-4 without detectable IL-2. As noted above, Con A activation of Fr. III cells sorted from unprimed mice resulted in IL-4 but not IL-2 secretion. In contrast to this re-
CD4+ T cell subsets, Fr. I-IV (as shown and described in Fig. 1), were sorted by FACS from 3-mo-old BALB/c mouse spleen. 3 x 10^5 sorted cells were cultured with 6 x 10^5 irradiated (137Cs at 2,500 rad) C57BL/6 (B6) spleen cells in 200 µl culture medium (RPBI 1640 + 10% FCS + 50 µM 2-ME) in 96-well microtiter plates. Supernatant from 4-d culture was tested for G. F. secretion. In contrast with Fr. I and II cultures, which showed aggregation and proliferation of cells, cultures of Fr. III and IV did not show any proliferation and all cells eventually died within 7 d.

The lack of detectable IL-2 is not due to inhibition of IL-2 activity in the bioassay by other secreted factor(s) in the supernatant since direct addition of IL-2 results in detection of the expected levels of IL-2 (data not shown). Therefore, we assume that repeated in vitro antigenic stimulation results in the expansion of specific mature T cell clones and less "bystander" activation, culminating in a cell population comparable with the Th2-type CD4+ cloned lines that synthesize IL-4 but not IL-2 (3). Since we generated memory T cell populations in bulk culture, IL-4 secretion is evidently a general property of CD4+ memory cells for both types of induction systems used here. This finding extends an earlier report of Powers et al. (8), who found an increased frequency of IL-4 secreting cells after in vitro restimulation with the antigen KLH. While our population study cannot exclude the possibility that the IL-4-secreting memory fractions arose from a minor precommitted subpopulation present in Fr. I, such cells would have to be a very minor component since IL-4 secretion is undetectable after polyclonal activation of this fraction. Thus, we favor the alternative hypothesis, that cells secreting IL-2 alone (in Fr. I or II) switch on maturation to screening IL-4 alone (and Fr. III or IV phenotype).
FIGURE 2. Transition from dominant IL-2 secretion to exclusive IL-4 secretion in culture induced by antigen restimulation. (A) Fr. III cells sorted from KLH-primed mice were antigen stimulated as described in Fig. 1. On day 7, cultured cells were washed and recultured with fresh medium and antigen; this was repeated on the days indicated by arrows. (B) Primary BALB/c anti-B6 MLR was initiated by culturing BALB/c spleen cells and irradiated B6 spleen cells (2 x 10^6 cells/ml each). On day 3, large CD4+ cells showing Fr. IV phenotype were sorted by FACS and restimulated as described in Fig. 1. Such secondary MLR cells were repeatedly stimulated in vitro. (C) Sorted Fr. I cells were used for the primary MLR and resorted to obtain Fr. IV cells on day 13, then restimulated. Supernatant from day 1 (and day 4) after each restimulation was tested for G.F. secretion. After several restimulations, G.F. activity of all supernatants was no longer inhibited by anti-IL-2, whereas anti-IL-4 inhibited completely. The results presented are representative data. In six such experiments the time for transition from IL-2 dominance to IL-4 varied somewhat, but was always observed.

Whereas both types of CD4+ memory cells generated herein produced IL-4, it remains to be determined whether factors or cells not present in our cultures of sorted cells (or different antigen stimulation) might alter the pathways of maturation seen here. Nevertheless, our results emphasize the dynamic nature of peripheral CD4+ cells, both in terms of phenotype and lymphokine production. We further suggest that different modes of antigen presentation may act on distinct subsets of memory T cells. Generation of two CD4+ memory populations together with observation of comparable in vivo fractions (which differ in activation; reference 4) provide an opportunity to study such differences.

Summary
Phenotypic and functional alteration of murine CD4+ T cells after antigenic stimulation was studied using two anti-T cell mAbs recently described that define four distinct T cell subsets. Activation of T cells resulted in the permanent loss of 3G11
expression. However, two phenotypically distinct memory T cell populations were established depending on the system used; whereas those for anti-KLH antibody response were enriched in the fraction expressing 6C10 (Fr. III), memory T cells for the allogeneic MLR lacked such expression (Fr. IV). Furthermore, successive stimulation with antigen in vitro resulted in secretion of IL-4 without detectable IL-2. This alteration of phenotype and interleukin secretion was also demonstrable when starting with 3G11+6C10- cells (Fr. I), the fraction that secretes IL-2 exclusively upon activation.

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