Characterization of Antigen-specific CD4+ Effector T Cells In Vivo: Immunization Results in a Transient Population of MEL-14-, CD45RB- Helper Cells that Secretes Interleukin 2 (IL-2), IL-3, IL-4, and Interferon γ

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Summary

In previous studies we demonstrated that, following activation by mitogens or alloantigens, helper T cell precursors proliferate and differentiate in vitro to produce a population of effector cells that secrete high titers of lymphokines upon restimulation. In this report, we demonstrate that a similar effector population develops in vivo following primary antigen stimulation. When restimulated with specific antigen in vitro, CD4+ T cells from mice primed 5 to 7 days previously by subcutaneous administration of keyhole limpet hemocyanin (KLH) in adjuvant, produced high levels of interleukin 2 (IL-2), IL-4, and IL-3, and little or no interferon γ (IFN-γ) or IL-5. The effector T cells provided excellent helper activity for in vitro antibody responses of 4-hydroxy-5-iodo-nitrophenyl acetic acid-primed B cells with the production principally of the immunoglobulin G₁ (IgG₁) and IgM isotypes, small quantities of IgG3, and no detectable IgG2a, or IgG2b. Antigen-specific secretion of IL-2, IL-3, and IL-4 by in vivo effectors was detectable by 12 hours following in vitro restimulation. IFN-γ and IL-5 were not detected until 48 and 72 hours of culture, respectively, and low levels of these lymphokines were produced. Lymphokine production by primed CD4+ T cells could be induced as early as 3 days following immunization, peaked on day 5, and declined thereafter. The kinetics of in vivo appearance of effector CD4+ T cells that produce lymphokines upon restimulation in vitro were similar for each of the lymphokines examined.

Mice depleted of precursor CD4+ T cells by adult thymectomy exhibited limited capacity to generate lymphokine secreting CD4+ T cells in response to primary immunization with KLH, suggesting that the majority of lymphokine producing T cells arise from short-lived and/or precursor cells. Separation of CD4+ T cells from KLH-primed mice on the basis of expression of the lymph node-specific homing receptor, MEL-14, revealed that antigen-specific production of IL-2, IL-3, IL-4, and IFN-γ was exclusively associated with the MEL-14- subset of CD4+ T cells. Separation on the basis of CD45RB expression, demonstrated that antigen-specific lymphokine production was primarily associated with the minor CD45RB- population, which has been previously associated with memory activity. Our results indicate that primary in vivo immunization leads to the development of a transient population of helper-effectors with a unique phenotype that can produce large quantities of lymphokines and mediate excellent helper activity for B cells. This population may be of critical importance for in vivo regulation of the immune response.

The immune response of an animal to an antigen that it has not previously encountered depends upon the statistically unlikely interaction of two rare antigen-specific cells: a helper T cell and a B cell which must each recognize foreign determinants on the same molecule. It has been suggested that the CD4+ helper T cell and B cell initially encounter antigen separately and then selectively migrate to specialized areas in secondary lymphoid organs where they have an increased likelihood of engaging in the interactions necessary for an effective B cell response. The response of the B cell depends both on the direct interaction of the B cell with the T cell and on the lymphokines produced by the T cell. Optimal B cell proliferation and differentiation into Ab-secreting cells requires several lymphokines produced

by helper T cells including IL-2, IL-4, IL-6, and perhaps IFN-γ (1). The generation of isotype switch in B cells requires quite high concentrations of IL-4 or IFN-γ for the respective switch to IgG1/IgE versus IgG2a. Paradoxically, despite the obvious importance of such lymphokines, naive CD4$^+$ T cells are able to synthesize and secrete only IL-2 in direct response to stimulation (2) and even resting memory CD4$^+$ T cells make relatively low quantities of lymphokines other than IL-2 (3–5) in comparison to cloned T cell lines which are very effective helpers and make prodigious quantities of lymphokines such as IL-4, IL-5, IL-6, and IFN-γ (1, 6).

Recently, we found that CD4$^+$ T cells with the phenotype and characteristics of naive precursor cells, proliferate and differentiate in vitro in response to alloantigens or mitogens to generate a population of cells in 3–4 d which, when restimulated secretes very high titers of IL-4, IL-5, and IFN-γ (3). We call these cells effectors by analogy with CTL development. If such cells are also generated during an in vivo in response to antigenic challenge and relocate appropriately, they could potentially act as very efficient populations of helpers for B cells during the primary immune response and could also mediate isotype switch. Therefore, we undertook an investigation of whether an effector population secreting lymphokines in addition to IL-2 would develop in vivo in response to immunization with conventional antigens and sought to determine its origin, lymphokine secretion profile, kinetics of development, and phenotype.

Previous studies of the regulation of lymphokine production by T cells have often used in vitro models of polyclonal activation and/or cloned in vitro cell lines. Therefore, very little is known about the characteristics and phenotype of lymphokine-secreting helper T cells that develop in vivo during an immune response. In addition, many of the previous studies investigating the development of antigen-specific CD4$^+$ T cells from immunized animals have used limiting dilution analysis combined with in vitro selection to generate clones of T cells (4, 7–10). Because lymphokine production is examined only after additional in vitro expansion, the kinetics of the response and phenotype of the cells which develop during primary immune responses in vivo have not generally been addressed. Several reports have stressed that IL-4 production requires one or more rounds of restimulation (3–5, 11, 12), although recent studies do suggest that both IL-2 and IL-4 production may occur in vivo during the first 5 d following exposure to antigen (13) and high levels of IL-4 production have been detected following infection with parasites (14, 15).

In this report, we examine the kinetics of in vivo development of antigen-specific CD4$^+$ T cells which arise in response to primary immunization with KLH and characterize the lymphokines that are produced, the cells that produce them, and the cells from which they were derived. We find that antigen-specific CD4$^+$ T cells with the capacity to mediate helper activity and to produce high titers of lymphokines, including IL-4, in response to restimulation develop after priming with a standard immunization regimen with similar kinetics to lymphokine secreting effectors generated in vitro. Development of CD4$^+$ effector cells in response to

a primary injection of antigen is dependent upon a population of precursor T cells that is largely depleted in adult thymectomized mice. We also examine the surface phenotype of the antigen-specific effector population in terms of expression of MEL-14, the lymph node specific homing receptor that is down-regulated in vitro following polyclonal activation of T cells (16), and of CD45RB(23G2), which appears to distinguish virgin from memory CD4$^+$ T cells (17) and can also be down-regulated following in vitro activation of CD4$^+$ T cells (18). These studies provide detailed characterization of effector helper T cells able to secrete high titers of lymphokines that are generated in vivo during an immune response.

Materials and Methods

Mice. BALB.B and C57BL/6, and (C57BL/6 × DBA/2)F$_1$ hybrid (BDF$_1$) mice were bred in our own facility from pedigreed breeders obtained from the Jackson Laboratories (Bar Harbor, ME). Adult mice of the same age were used in individual experiments.

Reagents. KLH was purchased from Calbiochem (La Jolla, CA), OVA, pigeon cytochrome C (Cyt C),[1] and BSA were obtained from Sigma Chemical Co. (St. Louis, MO). NIP (4-hydroxy-5-iodo-nitrophenyl acetic acid) (Cambridge Research Biochemicals, Valley Stream, NY) was conjugated to OVA, KLH, CytC, or BSA as previously described (19). Medium for all in vitro cultures was RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 200 μg/ml penicillin, 200 μg/ml streptomycin, 4 mM L-glutamine, 10 mM HEPES, 5 × 10^{-5} M 2-ME, and 7% FCS (Hyclone Laboratories, Logan, UT).

Immunizations. To generate primed CD4$^+$ T cells, mice were immunized subcutaneously at the base of the tail with 250 μg KLH or OVA emulsified in either complete or incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI). Draining periaortic lymph nodes were removed at various times after immunization and used as a source of T cells. Hapten-primed B cells were obtained from mice that were initially immunized by i.p. injection of 100 μg NIP-OVA precipitated with 1.8 mg alum together with 10^9 heat-inactivated *Bordetella pertussis* organisms. After 2 to 4 mo, mice were boosted intravenously with 20 μg soluble NIP-OVA and B cells were isolated from the spleens 10 to 14 d later.

Antibodies. The antibodies for depletion of cell populations have been previously described (3) and include anti-Thy-1.2 (F7D5 and HO.13.14), anti-Ly 2.2 (HO.2.2), anti-L3T4 (RL172.4), and anti-Class II (D3.137) (a kind gift of Dr. Susan Tonkonogy, North Carolina State University, Charleston, NC). Fluoresceinated (FL)-anti-Ly 2 (anti-CD8) and phycoerythrin labeled (PE)-GK1.5 (anti-CD4) were obtained from Becton Dickinson and Co. (Mountain View, CA). Pgp-1 (anti-CD44) (20), 23G2 (a kind gift of Dr. Ellen Puré, Rockefeller University, New York, NY) (18) and MEL-14 (21) cell lines were cultured to generate antibodies used in indirect immunofluorescence with FL-RG7/9/1 (mouse anti–rat κ) to detect subsets of CD4$^+$ T cells. Goat anti–mouse isotype-specific antibodies labeled with horseradish peroxidase for the ELISA assay of NIP-specific antibody from in vitro secondary anti-hapten responses were purchased from Southern Biotechnology Associates (Birmingham, AL). The following antibodies were obtained as ascites

from nude mice as previously described (22): 11B11: anti–IL-4; 7D4 and PC.61: anti–IL-2R, TRFK4 and TRFK5: anti–IL-5, and R46A2 and XMG1.2: anti–IFN-γ.

Recombinant Lymphokines. Human rIL-2 was obtained from Cetus Corporation (Emeryville, CA). Murine rIL-3 was obtained from Genzyme (Boston, MA). Murine rIFN-γ was obtained from Amgen Biologicals (Thousand Oaks, CA). rIL-4 and rIL-5 were obtained from the culture supernatants of the X63.Ag8-653 line that was transfected with the murine cDNA for IL-4 or for IL-5, respectively (23). The IL-4 and IL-5 secreting lines were the kind gift of Dr. Fritz Melchers (Basel Institutes for Immunology, Basel, Switzerland).

Thymectomy. BDF₁ or C57BL/6 mice were thymectomized by suction at 8 wk of age or older. At the time of sacrifice, mice were checked for thymic remnants and spleen cells were stained for cells that coexpress CD4 and Pgp-1 which are enriched in thymectomized mice (3). Thymectomized mice were compared to untreated control mice of the same age and strain.

Preparation of CD4⁺ T Cells. Periaortic lymph node cells from mice immunized with KLH or OVA in adjuvant were depleted of B cells by treatment at 2×10^7 ml with 0.5 µg/ml biotinylated rat anti–mouse κ (Pharmingen, San Diego, CA) for 30 min at 4°C, followed by incubation with Streptavidin-coated magnetic particles (Advanced Magnetics, Cambridge, MA) at a 40:1 ratio for an additional 30 min at 4°C. Surface Ig positive cells were depleted by three 10-min cycles of exposure to a magnetic field. The remaining lymph node cells were further enriched for CD4⁺ T cells by treatment with anti-J11D, D3.137, and Ly 2.2 antibodies and a combination of guinea pig (Gibco Laboratories, Grand Island, NY) and rabbit complement (Accurate Chemical and Scientific Corporation, Westbury, NY) to deplete CD8⁺ T cells and residual B cells. The resulting populations were 80 to 90% CD4⁺ T cells.

Magnetic Activated Cell Sorter (MACS) Separation of CD4⁺ T Cells. Subsets of CD4⁺ T cells were prepared by magnetic separation with a MACS column (Miltenyi Biotech, Germany) (24). Enriched CD4⁺ T cells were sequentially stained with either MEL-14 or CD45RB (23G2), biotinylated-RG7/9/1, Fl-Strepavidin, and biotinylated magnetic particles, according to the manufacturer's instructions. The positively staining population was retained by the MACS column in the presence of the magnetic field, while the negative population was eluted. Populations were checked for purity by flow cytometry and reincubated on the column as necessary to achieve maximum separation of the positive and negative subsets. Due to washing of the columns during retention of the positive population, as well as sequential passages of both the positive and negative populations over the column, generally 30 to 50% of the initial stained population was recovered following the isolation procedure.

Preparation of B Cells and APC. T cells were depleted from normal or antigen-primed spleen cell populations by treatment with a cocktail of directly cytotoxic anti-T cell reagents (two anti-Thy 1.2 antibodies, anti-CD4, and anti-CD8) and complement. The resulting populations consisted of >95% surface Ig⁺ cells. T-depleted spleen cells from normal animals were used as a source of APCs for the induction of lymphokine production by antigen-primed CD4⁺ T cells. In some experiments, the APCs were pulsed with antigen by overnight culture in the presence of 100 µg/ml of either OVA or KLH. T-depleted spleen cells from NIP-OVA primed mice were used as a source of antigen-primed B cells to assay helper activity of KLH-primed CD4⁺ T cells.

Flow Cytometry. Lymphoid populations (10⁶) were stained by incubation with antibody for 30 min at 4°C in PBS containing 5% FCS and 0.5% NaN₃, washed, and fixed with 1% methanol-free formaldehyde in PBS. In the case of double staining, cells were incubated with an unlabeled first step antibody, followed by FL-RG7, and finally with PE-conjugated anti-CD4. 5×10^3 cells were analyzed using a FACSCAN flow cytometer (Becton Dickinson and Co.).

In Vitro Stimulation of Lymphokine Production by CD4⁺ T Cells. CD4⁺ lymph nodes from KLH-primed mice were cultured in either 0.75 ml or 1.5 ml volumes in 48 or 24 well plates, respectively, at 10⁶/ml (or at concentrations indicated in the text for individual experiments) together with 10⁶/ml APC. The cultures were immunized with 75 µg/ml KLH, or an identical concentration of the irrelevant antigen, OVA. In some experiments antigen-pulsed APC were used (see above). Culture supernatants were harvested after 24 to 36 h, unless otherwise indicated. Culture supernatants were stored at −20°C before testing for lymphokine content.

Assays of Lymphokine Production. The bioassays used for detection of lymphokines in the culture supernatants of antigen-primed CD4⁺ T cells have been previously described (3, 22) and are briefly summarized below. In all assays, serial two-fold dilutions of test supernatants, starting at 1:10, were added to triplicate cultures of indicator cells in 0.1 ml volumes in 96-well plates (Costar, Cambridge, MA). The data are quantitated from standard curves generated using serial two-fold dilutions of control recombinant lymphokines. The data are expressed as U/ml, where 50% of the maximal response represents the activity of 1 U. U/ml are calculated from the reciprocal of the dilution that gives half maximal activity, divided by the culture volume. For proliferation assays, indicator cells were cultured with test supernatants or control lymphokines for 48 h and pulsed with ¹²⁵I-UdR during the last 16 h of incubation. Cultures were harvested onto glass fiber filter paper using a multiple sample harvester, and counted with a gamma counter.

IL-2 and IL-4 in T cell culture supernatants was detected using the NK cell line, which responds to both lymphokines. To detect IL-2, IL-4 activity was inhibited by the addition of the 11B11 anti–IL-4 antibody at 1.0 µg/ml. To detect IL-4, IL-2 activity was inhibited by the addition of 1.0 µg/ml each of two anti–IL-2R antibodies, PC.61 and 7D4. The anti–IL-4 and anti–IL-2R antibodies together completely blocked proliferation in response to test supernatants. In this assay, maximum levels of NK cell proliferation were obtained with 2 U/culture (3 pg) of rIL-2, and 3 U/culture (45 pg) of rIL-4. The 11B11 antibody blocks up to 30 U/culture of IL-4 without affecting the IL-2 response, and the PC.61 and 7D4 antibodies block up to 12 U/culture of IL-2 without inhibiting the IL-4-response (not shown).

IL-3 in test culture supernatants was detected by the proliferation of the 32Dc15 line, which does not respond to other lymphokines under the conditions of our experiments (3). Maximum proliferation was obtained with 3 U/culture (3pg) of rIL-3 as defined by Genzyme.

IL-5 and IFN-γ were detected by ELISA assay. Polyvinyl chloride 96-well plates (Falcon, Oxnard, CA) were coated overnight with 1.0 µg/ml TRFK.5 anti–IL-5 or R46A2 anti–IFN-γ antibodies, respectively, in PBS pH 7.4 and blocked with PBS containing 10% FCS, 1% BSA, 0.05% Tween 20 for 30 min at 37°C. The plates were washed and incubated with dilutions of culture supernatants and control rIL-5 and rIFN-γ for 1 h at 37°C. The plates were then incubated sequentially with: biotinylated TRFK.4 anti–IL-5 or XMG1.2 anti–IFN-γ for 1 h at room temperature; peroxidase-labeled streptavidin for 1 h at room temperature; and 100 µg/ml O-phenylenediamine dihydrochloride and 200 µg/ml urea hydrogen peroxide in citrate phosphate buffer for 30 min at room temperature. The plates were washed between steps. The reac-

tion was stopped by the addition of H_2SO_4 in a final concentration of 5%, and the plates were read at 492 nm using a Titertek Multiskan MCC/340 ELISA reader (Flow Laboratories, McLean, VA). IL-5 and IFN-γ present in individual culture supernatants were quantitated by comparison to standards containing rIL-5 and rIFN-γ. 1 U of rIFN-γ as defined by Amgen Biologicals equals the quantity required to neutralize viral destruction of L-929 cells by 50% and the limit of detection in the IFN-γ ELISA is 0.5 ng/ml (5 U/ml). 1 U of X63.Ag8-653 IL-5 is equivalent to 1 U of rIL-5 as defined by Genzyme as the amount required to induce half maximal proliferation of dextran sulfate-stimulated B cells and the limit of detection in the IL-5 ELISA is 2 ng/ml (2 U/ml).

Measurement of Helper Activity of In Vivo Primed CD4+ T Cells. T-depleted spleen cells from NIP-OVA primed mice were culture in duplicate at 1.25 × 10⁶/ml together with KLH-primed CD4+ T cells, as indicated in the text, in 0.7 ml volumes in 48-well plates (Costar). The cultures were immunized with 0.03 μg/ml NIP-KLH, or with the same doses of NIP-OVA or NIP-Cyt C. After 7 d, the culture supernatants were harvested and assayed for the presence of NIP-specific antibodies with an antigen and isotype specific ELISA using NIP-BSA–coated plates and horseradish peroxidase-labeled goat anti–mouse antibodies specific for IgM, IgG1, IgG3, IgG2a, and IgG2b. Anti-NIP antibody present in individual culture supernatants was quantitated in comparison to an anti-NIP sera generated in BALB.B mice. Standard curves were generated using serial two-fold dilutions of the sera starting at 1:400 for IgM, IgG3, IgG2a, and IgG2b, and at 1:3200 for IgG1. These dilutions for the individual isotypes were assigned a value of 1,000 U/ml.

Results

In Vivo Induction of Antigen-specific CD4+ Lymphokine-secreting T Cells. To study the in vivo induction of lymphokine producing T cells, mice were immunized s.c. with KLH in CFA. 5 to 7 d after immunization, CD4+ T cells were enriched from the draining periaortic lymph nodes and cultured at varying concentrations together with 10⁶/ml mitomycin-treated, T cell-depleted spleen cells from normal animals as a source of APC, and with either OVA or KLH. The supernatants were harvested after 24 h and tested for the presence of IL-2, IL-3, IL-4, IL-5, and IFN-γ. Stimulation with KLH resulted in production of IL-2, IL-3, and IL-4 by the primed CD4+ T cells as shown in Fig. 1 *A*. Stimulation with OVA did not lead to lymphokine production, indicating both that the cells required restimulation to produce lymphokines and that the response was specific for the priming antigen, KLH. IFN-γ titers in the same supernatants were less than 20 U/ml and IL-5 was less than 5 U/ml (not shown). Comparable results have been obtained in over five separate experiments. Similar results were obtained when mice were primed and challenged with OVA in CFA, and when antigen was administered in IFA although the magnitude of the responses were less (not shown). No lymphokine production was obtained in the absence of added APC, and no detectable lymphokines were produced by the APC population alone in response to KLH (not shown). In each bioassay, all of the supernatant activity was blocked by appropriate Ab to the relevant test lymphokine, demonstrating the specificity of the assays (not shown). KLH at doses from

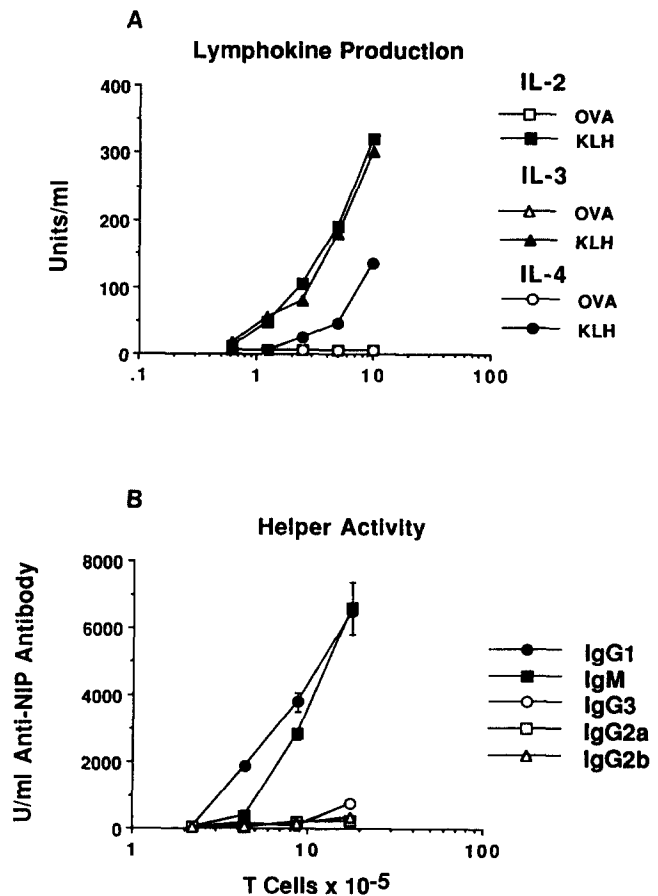## KLH-Specific CD4+ T Cells Generated by In Vivo Immunization

Figure 1. IL-2, IL-3, and IL-4 production by CD4+ KLH-primed T cells. (*A*) CD4+ lymph node T cells from BDF₁ mice primed 5 d previously with KLH in CFA were highly enriched for CD4+ T cells as described in the Materials and Methods. Before culture the cells were stained by indirect immunofluorescence with the indicated antibodies and followed by FITC-RG7/9/1 (anti–rat κ chain antibody) and contained 90% GK1.5 (CD4+) cells, 7.4% Ly2.2 (CD8+) cells, and 11.4% Bet-2 (sIgM+) cells. The indicated numbers of cells were cultured with 10⁶ APC and either KLH or OVA. Culture supernatants were harvested at 24 h and tested for the presence of IL-2, IL-3, IL-4, IL-5 and IFN-γ as described in the Materials and Methods. Less than 20 U/ml of IFN-γ and 5 U/ml of IL-5 were detected (not shown). (*B*) Helper activity of KLH-primed CD4+ T cells. CD4+ lymph node T cells were isolated from BALB.B mice 5 d after immunization with KLH and combined in various concentrations with NIP-OVA primed B cells and either NIP-KLH or NIP-Cyt C. After 7 d, supernatants from duplicate cultures were harvested and assayed for anti-NIP antibodies using an isotype-specific ELISA as described in the Materials and Methods.

50–100 μg/ml were found to be optimum for stimulating lymphokine production (not shown).

It should be noted that immunization with CFA alone resulted in detectable levels of IL-2 and IL-3 (that typically comprise 10–30% of the response), but not of IL-4, IL-5, or IFN-γ in cultures stimulated with KLH-pulsed APCs (not shown). Since no detectable lymphokines were produced by

either adjuvant- or KLH-primed CD4$^+$ T cells to OVA, these results suggested that CFA-priming generates a small population of T cells crossreactive with KLH. Indeed, when mice were primed with IFA, the adjuvant effect in terms of IL-2 and IL-3 production to KLH was eliminated (not shown). The majority of the lymphokine producing activity was present in the draining periaortic lymph nodes, although small responses could also be obtained with CD4$^+$ T cells isolated from inguinal lymph nodes (our unpublished observations).

We examined the capacity of KLH-primed CD4$^+$ T cells to mediate helper activity in secondary in vitro anti-hapten responses. CD4$^+$, KLH-primed T cells were isolated at 5 d after immunization and combined with T-depleted spleen cells from mice that were primed and boosted with NIP-OVA as a source of B cells. The cultures were immunized with NIP-KLH, and supernatants were harvested after 7 d of incubation. The presence of NIP-specific antibody was measured by an antigen- and isotype-specific ELISA. The results, shown in Fig. 1 B, indicate that the short-term primed CD4$^+$ T cells provided excellent helper activity for B cells and that IgG1 and IgM are predominant isotypes of anti-NIP antibody produced under the conditions of the experiment. The presence of helper activity for these isotypes is dependent upon

priming: no antibody production was observed when cultures were immunized with NIP conjugated to the irrelevant carrier, Cyt C (data not shown). The KLH-primed, CD4$^+$ T cell used in this experiment were tested for lymphokine production and 1.5 × 10^6 cells secreted 530 U/ml IL-2 and 500 U/ml IL-4.

Kinetics of In Vitro Lymphokine Secretion by KLH-primed CD4$^+$ T Cells.   We examined the in vitro kinetics of lymphokine production following specific restimulation of KLH primed CD4$^+$ T cells. The results shown in Fig. 2 demonstrate that IL-2, IL-3, and IL-4 could be detected in supernatants by 12 h following restimulation with KLH in culture. In contrast, IFN-γ and IL-5 were not detected until 48 and 72 h, respectively, and only low levels of these lymphokines were observed (note the different scales of the graphs). Since low levels of IFN-γ can be detected by the ELISA, and IFN-γ could be readily measured in 36-h supernatants when the lymphokine-secreting population was enriched (see below), the apparent delayed production of this lymphokine presumably reflects accumulation of sufficient levels for detection. In contrast, IL-5 was not detected in supernatants collected at 48 h or earlier, even when the lymphokine-secreting population was enriched (see below) probably because the ELISA

Kinetics of In Vitro Lymphokine Production
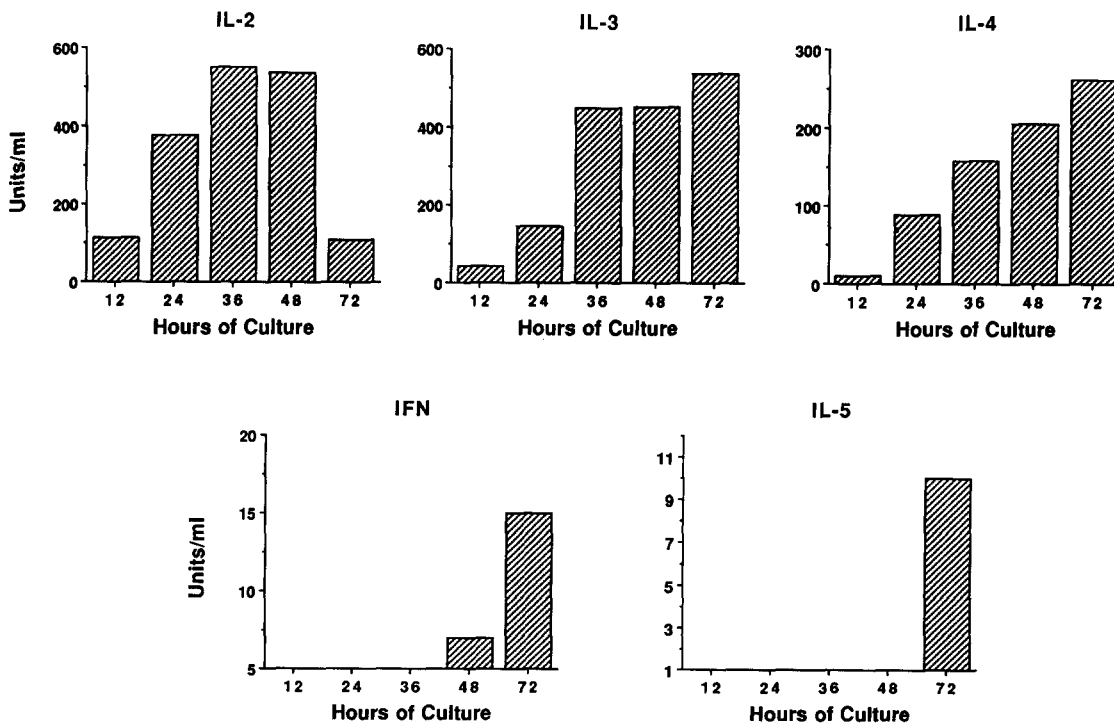### by KLH-Specific, CD4$^+$ T Cells

Figure 2.   In vitro kinetics of lymphokine production by KLH-primed CD4$^+$ T cells. CD4$^+$ lymph node T cells were isolated from C57BL/6 mice from mice primed with KLH 5 d previously, and cultured with APC and KLH as described for Fig. 1. Culture supernatants were harvested at the indicated times, and assayed for the presence of IL-2, IL-3, IL-4, IFN-γ, and IL-5.

assay for IL-5 is relatively insensitive (see Materials and Methods). The results indicate that in vivo priming with KLH generates a population of CD4+ T cells that secretes predominately IL-2, IL-3, IL-4, and IFN-γ and possibly low titers of IL-5, regardless of the time after in vitro restimulation. There was a drop in IL-2 found in culture supernatants at 72 h, presumably as a result of IL-2 uptake by responding cells. In subsequent experiments, lymphokines were harvested from 12 to 36 h to minimize the influence of uptake on the results.

Kinetics of In Vivo Development of KLH-specific Lymphokine-secreting T Cells. To further characterize the development of antigen-specific lymphokine secreting T cells which develop in vivo, lymphokine production by enriched CD4+ T cells, isolated from the lymph nodes of KLH-primed animals at various times after immunization was determined. Supernatants were collected from replicate cultures after 12 and

24 h and assayed for IL-2, IL-4, IL-3, IL-5 and IFN-γ. The results for 24-h supernatants shown in Fig. 3 are representative of three of the experiments in which the kinetic development of CD4+ T effector cells was determined. 12-h supernatants had lower levels of lymphokine activity but showed the same patterns (not shown). Lymphokine secretion was detectable as early as 3 d after immunization, peaked at days 5 to 7, and declined steadily thereafter. Similar kinetics were observed for the development of CD4+ T cells with the capacity to produce IL-2, IL-3, IL-4, and IFN-γ and no IL-5 was detected under the conditions of these experiments. Lymphokine-secreting CD4+ T cells that develop in response to i.v. administration of SRBC exhibit similar kinetics, but produce primarily IL-2 and IL-3, moderate amounts of IL-4, and IFN-γ and no detectable IL-5 (not shown).

Antigen-specific CD4+ Lymphokine–producing Cells Develop from Short-lived Precursors. In previous studies, we demon-

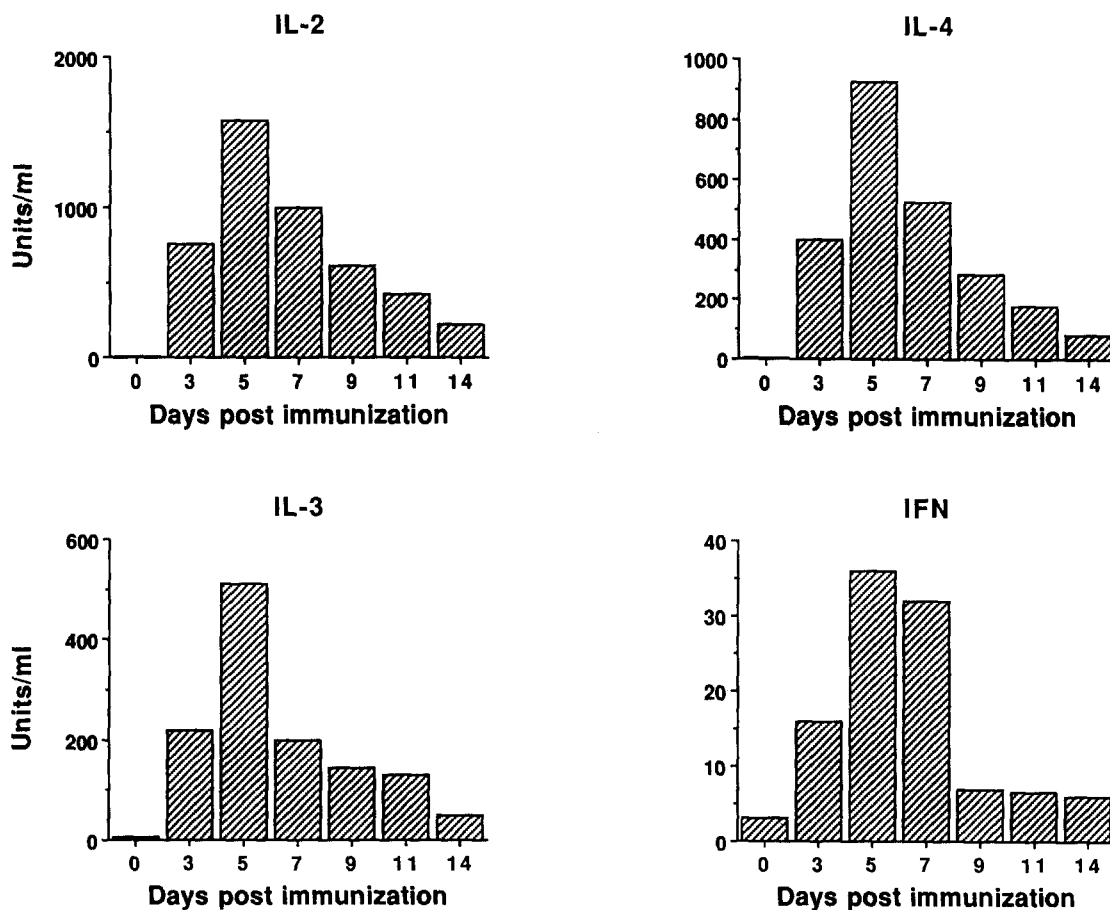## Kinetics of In Vivo Development of KLH-Specific Effector CD4+ T Cells

Figure 3. Kinetics of lymphokine production by CD4+ KLH-primed T cells. CD4+ lymph node T cells were isolated from BALB.B mice at the indicated times after immunization with KLH in CFA and cultured at 10⁶/ml with an equal number of APC and either OVA (data not shown) or KLH. Culture supernatants were harvested from replicate cultures at either 12 or 24 h and tested for IL-2, IL-3, IL-4, IL-5, or IFN-γ activity as described for Fig. 1. No lymphokines were detected in the supernatants of KLH-primed CD4+ cells cultured with OVA (not shown). No IL-5 was detected (not shown).

strated that CD4$^+$ T cells activated in vitro with mitogens or allogeneic cells develop in the presence of exogenous lymphokines (IL-2 and IL-4) into effector cells that produce high titers of lymphokines (IL-3, IL-4, IL-5, and IFN-γ) upon restimulation (3, 22). Development of the in vitro effector population depends upon CD4$^+$ T cells that express low levels of the homing receptor, Pgp-1, and are depleted from the spleen by adult thymectomy. To determine if the antigen-specific CD4$^+$ T cell population that is induced in vivo is

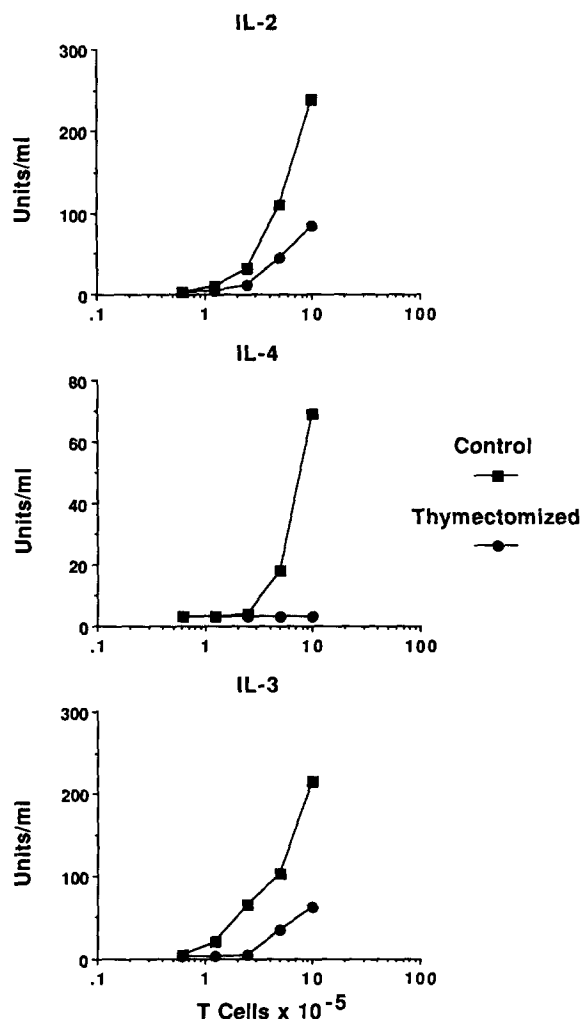### Development of KLH-Specific Effector CD4$^+$ T Cells is Reduced in Thymectomized Mice

Figure 4. Effect of thymectomy on lymphokine production by CD4$^+$ KLH-primed T cells. CD4$^+$ lymph node T cells were isolated from control and thymectomized C57BL/6 mice that were immunized 5 d previously with KLH and cultured at 10^6/ml with an equal number of APC, and either OVA (data not shown) or KLH. Culture supernatants were harvested at 24 h and tested for the presence of lymphokines. Shown are IL-2, IL-3, IL-4 produced by CD4$^+$ T cells from control versus thymectomized mice. Low levels of IFN-γ production (8 U/ml) were detected only from the highest concentration of cells from control animals. No IL-5 was detected, and no lymphokines were produced in cultures immunized with OVA (not shown).
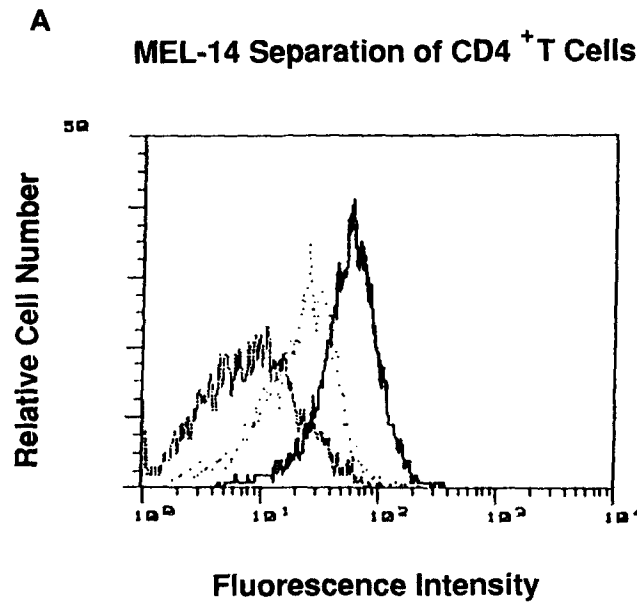
derived from similar precursor T cells, we compared the development of antigen-specific CD4$^+$ lymphokine secreting cells in control (age-matched) and adult thymectomized mice (6 mo after thymectomy).

Although no differences in Pgp-1 expression by CD4$^+$ lymph node cells from normal and KLH-primed mice could be detected (not shown), the CD4$^+$ T cells from adult thymectomized mice exhibited dramatically increased levels of Pgp-1: CD4$^+$ T cells from control animals displayed a single broad peak of fluorescence with a mean log fluorescence intensity (MFI) of 16.6, while those from thymectomized mice exhibited 2 peaks of fluorescence with MFI values of 35.4 and 275.6. CD4$^+$ T cells comprised 38% of the lymph node population of control animals, and 15% of the lymph node cells from thymectomized animals. CD8$^+$ T cells and surface Ig$^+$ cells were comparable in control and thymectomized mice (19% vs. 26% Ly2$^+$, 17% vs. 22% Ig$^+$, respectively), confirming that thymectomy causes a selective reduction in CD4$^+$ T cells that express low levels of Pgp-1, and that presumably represent the naive CD4 precursor population.

We examined the effect of thymectomy on the in vivo generation of antigen-specific CD4$^+$ lymphokine secreting T cells by comparing in vitro lymphokine production by CD4$^+$ lymph node cells from control and thymectomized animals 5 d after immunization with KLH. The results shown in Fig. 4 demonstrate that CD4$^+$ T cells derived from thymectomized mice are deficient in the antigen-induced effectors that secrete IL-2, IL-3, and IL-4 in response to in vitro restimulation. Low levels of IFN-γ (8 U/ml) were detected in supernatants from the highest T cell concentration tested from control, but not thymectomized mice. When the approximately two-fold decrease in CD4$^+$ T cell recovery from thymectomized versus control animals is taken into account, the reduction in the capacity of thymectomized animals to generate lymphokine secreting effectors is even more pronounced. In addition, the residual CD4$^+$ T cells producing IL-2 and IL-3, but not IL-4 from thymectomized mice may in part represent an adjuvant activation of long-lived helper T cells or of residual precursor cells, since CFA was used for KLH immunization.

As expected from previous studies (3), CD4$^+$ T cells isolated from the spleens of the thymectomized KLH-primed mice exhibited significantly increased levels of Pgp-1, and were unable to generate lymphokine secreting effector cells in vitro to polyclonal stimulation with Con A (in the presence of APC, rIL-2 and rIL-4), but produced IL-2 in response to Con A plus PMA (not shown). The fact that CD4$^+$ T cells from thymectomized mice exhibit a reduced capacity to generate lymphokine producing T cells in response to either in vivo or in vitro stimulation suggests that the majority of lymphokine secreting effector cells are generated from short-lived, presumably naive precursor cells.

KLH-primed CD4$^+$ Lymphokine-secreting T Cells Are MEL-14$^-$. To further characterize the in vivo generated CD4$^+$ effector cells, we examined their expression of MEL-14, a lymph node-specific homing receptor that has been reported to be down-regulated shortly after polyclonal activation of

A

MEL-14 Separation of CD4 $^+$T Cells

Relative Cell Number

Fluorescence Intensity

B

MEL-14⁻, CD4⁺ T Cells Account for Lymphokine Production by KLH-Specific Effectors

IL-2

Units/ml

T Cells x 10^{-5}

IL-4

T Cells x 10^{-5}

IL-3

Units/ml

T Cells x 10^{-5}

IFN

T Cells x 10^{-5}
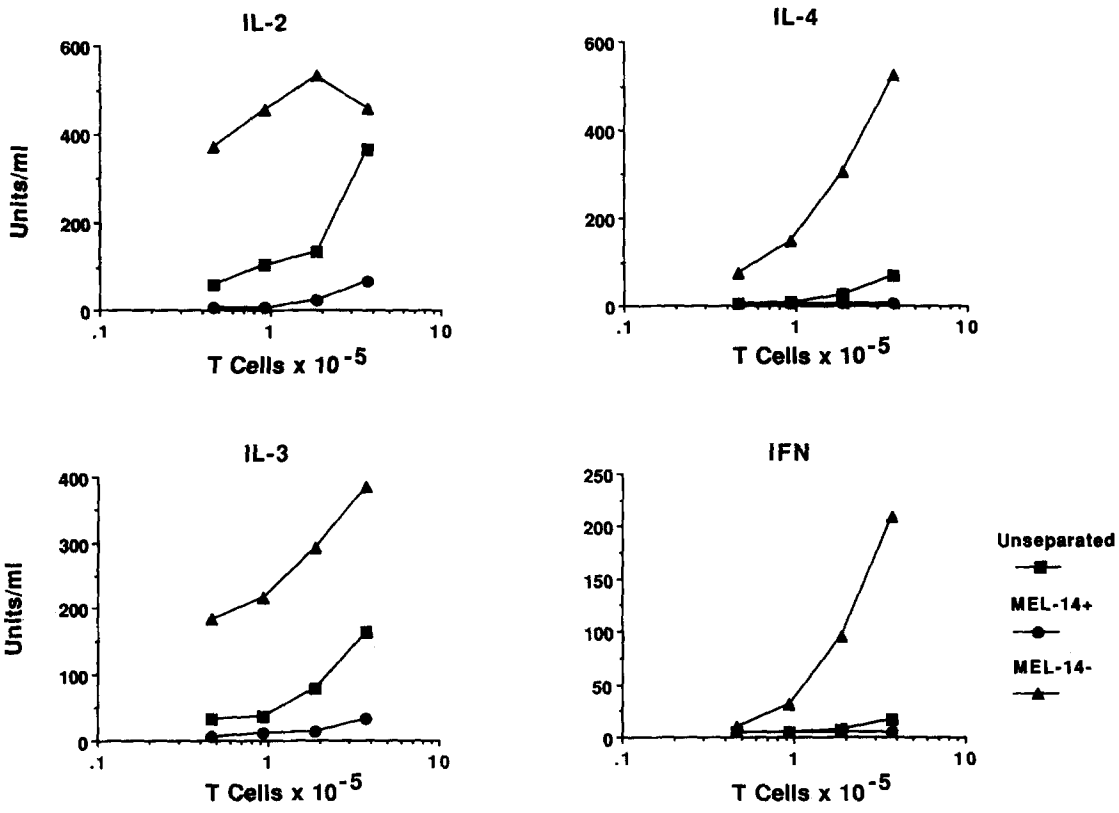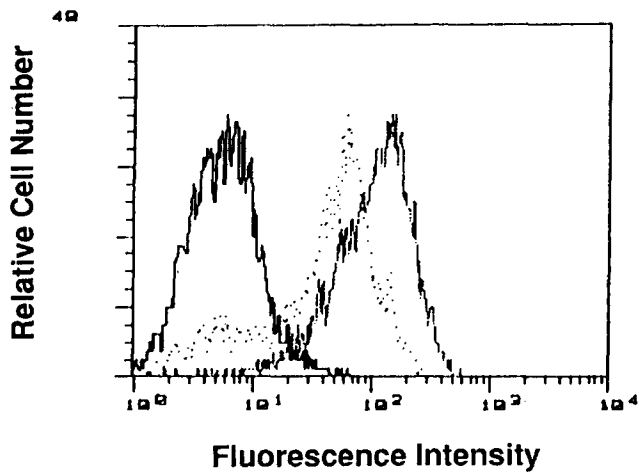
Unseparated
MEL-14+
MEL-14-

Figure 5. Separation of KLH-specific CD4⁺ lymphokine-secreting T cells on the basis of MEL-14 expression. (A) CD4⁺ lymph node T cells were isolated from mice primed 4 d previously with KLH. The cells were stained with MEL-14 and separated into positive and negative subsets using MACS as described in the Materials and Methods. The dotted histogram (center) represents the stained CD4⁺ population before separation. (B) MEL-14 positive and negative subsets were compared to stained, unseparated CD4⁺ T cells for the capacity to produce lymphokines in culture supernatants collected 36 h after in vitro restimulation with KLH-pulsed APC. No IL-5 was detected (not shown).

A

CD45RB Separation of CD4$^+$ T Cells

B

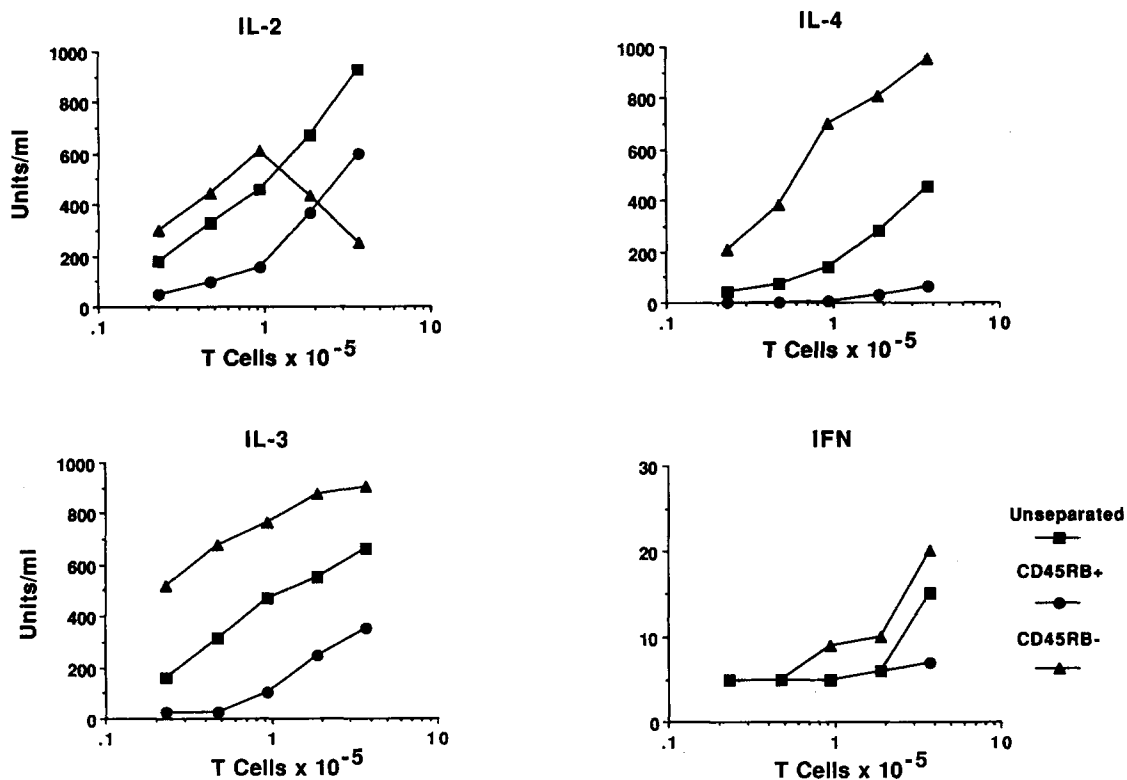## Enrichment of KLH-Specific Effectors in the CD45RB$^-$ Subset of CD4$^+$ T Cells

Figure 6. Separation of KLH-specific CD4$^+$ lymphokine secreting T cells on the basis of CD45RB expression. (*A*) CD4$^+$ lymph node T cells were isolated from mice primed 5 d previously with KLH. The cells were stained with 23G2 and separated into positive and negative subsets using MACS as described in the Materials and Methods. The dotted histogram represents the stained CD4$^+$ population before separation. (*B*) The CD45RB positive and negative subsets were compared to stained, unseparated CD4$^+$ T cells for lymphokine production in culture supernatants collected 36 h after in vitro restimulation with KLH-pulsed APC. No IL-5 was detected (not shown).

lymph node T cells in vitro (16). MEL-14 positive and negative subsets were isolated by magnetic (MACS) separation of CD4+ lymph node T cells from mice primed 4 d previously with KLH. Before separation, the lymph node CD4+ population consisted predominantly of MEL-14+ cells with moderate levels of staining (dotted histogram, Fig. 5 A). The MEL-14− subset represented less than 10% of the starting population but was enriched to 90% by the procedure, as shown in Fig. 5 A.

Lymphokine production by the MEL-14 positive and negative subsets in response to restimulation with KLH-pulsed APC is shown in Fig. 5 B. The data demonstrate that antigen-specific production of IL-2, IL-3, IL-4 and IFN-γ was exclusively a property of the MEL-14− cells. IL-5 was not detected. The MEL-14+ subset did not suppress the response of MEL-14− cells to KLH, but was capable of producing IL-2 and IL-3 in response to polyclonal activation with Con A (data not shown), indicating that these cells were not simply inactivated by staining with MEL-14. In addition, we have found no effect of MEL-14 staining on the function of CD4+ T cells in assays of helper function or proliferation (Atkins, G. G., L. M. Bradley, and S. L. Swain, unpublished results), CD4+ T cells with intermediate MEL-14 staining have not been examined but comparison with the unseparated population suggests that the great majority of lymphokine production can be attributed to the minor MEL-14− subset.

KLH-primed CD4+ Lymphokine-secreting T Cells Are Predominantly CD45RB−. To further characterize the antigen-specific CD4+ effector population generated in vivo, we examined expression of CD45RB (23G2). As shown in Fig. 6 A, unseparated CD4+ lymph node cells from KLH-primed mice (dotted histogram) contained predominately 23G2+, CD4+ T cells (78%). The positive and negative subsets were enriched to greater than 95% by magnetic separation. In response to in vitro restimulation with KLH-pulsed APC, the CD45RB− population secreted high levels of IL-2 as well as IL-3, IL-4, and was responsible for virtually all of the IL-4 and IFN-γ production (Fig. 6 B). In contrast, the CD45RB+ population secreted high levels IL-2 and moderate levels of IL-3, but not IL-4 or IFN-γ in response to antigen stimulation (Fig. 6 B). However, low numbers of CD45RB− cells produced much more IL-2 and IL-3 than the same number of CD45RB+ cells. No effect of the CD45RB staining was detected in mixtures of the positive and negative subsets, and both the CD45RB positive and negative subsets produced IL-2, following polyclonal activation with Con A (not shown). The data indicate that the majority of antigen-specific lymphokine secretion by CD4+ T cells from recently primed mice is associated with the CD45RB− subset.

Discussion

We have investigated the in vivo development of a population of antigen-specific helper-effector T cells and have characterized them in terms of the patterns and amount of lymphokines produced, kinetics of response, and surface phenotype. Several conclusions can be drawn from these studies. First, a transient population of CD4+ T cells which secretes high titers of IL-2, IL-3, and IL-4 develops in the draining lymph nodes within a few days of injection of antigen. The lymphokine secretion requires antigen-specific restimulation in vitro, and the population can provide excellent helper activity for B cells. Second, the effector population in this primary response is dependent upon, and likely derives from a short-lived population of T cells which is depleted following adult thymectomy and includes cells of naive phenotype. Third, the effectors are found exclusively in the minor MEL-14− population of lymph node CD4+ T cells from primed animals. Effectors which produce IL-4 and IFN-γ are contained exclusively within the CD45RB− subset of CD4+ T cells and IL-2 and IL-3 producing effectors are enriched in this population. A minor portion of IL-2 and IL-3 secretion is found among the CD45RB subset.

In this study we analyzed antigen-specific stimulation of lymphokine production by primed CD4+ T cells in vitro as well as the kinetics of the development of the responding population in vivo (Fig. 2 and 3). Our data do not suggest that IL-2 production precedes that of IL-4, as recently reported by Mohler and Butler following in vivo sensitization with picryl chloride (13). In our study, antigen-specific CD4+ T cells with the capacity to produce IL-2, IL-3, and IL-4 by 12 h following specific restimulation in vitro were apparent as early as 3 d after immunization with KLH, exhibited peak activity from 4–7 d, and rapidly declined, thereafter. Although IFN-γ was generally not detected in culture supernatants until 48 h, enrichment of the lymphokine-producing effector population on the basis of low expression of MEL-14 (Fig. 5 B) or CD45RB (Fig. 6 B) enabled detection of high titers of IFN-γ in 24 to 36 h supernatants. Since the ELISA assays used to detect IFN-γ and IL-5 (which detect 500 pg and 2,000 pg, respectively) are approximately 10–80 times less sensitive then the bioassays for IL-2, IL-3 and IL-4 (which detect 3 pg of lymphokine [IL-2, IL-3] to 50 pg [IL-4]), the low levels of INF-γ probably represent significant production. Because of the relative insensitivity of the ELISA, IL-5 may also be produced at low levels which are not being detected. Similar in vivo kinetics of effector development have been observed in the induction of T cells with the capacity to produce IL-4 after in vivo priming with goat anti–mouse δ (12). In addition, we found that effector cells that are generated in response to in vivo immunization with SRBC also exhibit peak activity within 4 to 7 d of priming (our unpublished observations). These results indicate that the activity of recently activated effector cells is transient, and suggests that the effector population may be short-lived and represents a primary helper T cell population that is generated in response to immunization.

We show here that antigen-primed CD4+ T cells produce readily detectable quantities of lymphokines, in addition to IL-2, upon in vitro restimulation with antigen (Fig. 1 and 2). In several previous studies, antigen-primed T cells produced only IL-2 in response to specific restimulation in vitro unless cells were expanded in limiting dilution or bulk cul-

tures by cycles of rest and restimulation with antigen (4, 5, 11). Several laboratories, including our own have also reported that lymphokine production which can be detected following polyclonal or alloantigen activation of freshly isolated CD4$^+$ T cells from normal, unimmunized mice is restricted primarily to IL-2 (3, 4, 8, 25), and have suggested that IL-4 production is associated with activated (large, low density) T cells (26). In vitro polyclonal restimulation of antigen-primed CD4$^+$ T cells elicits secretion of IL-3, IL-4, and IFN-γ in addition IL-2, analogous to the antigen-specific effector population described here (8, 12). Although in vivo generated KLH-specific effectors secreted readily detectable titers of IL-2, IL-3, and IL-4, and low levels of IFN-γ in response to in vitro restimulation with antigen, we did not assess lymphokine production by individual cells and thus cannot determine whether subsets analogous to Th-1 and Th-2 cells are generated, and/or whether cells with mixed phenotypes are represented. However, in other studies of helper T cell clones derived shortly after immunization with protein antigens in adjuvant, mixed patterns of lymphokine secretion were predominantly obtained (8–10).

A number of studies of polyclonal activated cells support the concept that naive T cells only can secrete IL-2 (2, 4, 12) in response to stimulation but that they can develop, in vitro, into cells which secrete large quantities of a greater array of lymphokines. In the response to KLH, we see little evidence of early IL-2 production presumably because of the low frequency of KLH-specific T cells in unimmunized animals. The large increase in IL-2 production, as well as production of IL-3 and IL-4, that requires in vitro restimulation and that depends on immunization, is consistent with the concept that the KLH-specific precursors have expanded greatly over the 5–7-d period and/or been recruited to the lymph node. Since the efficient development of Ab-secreting cells capable of production of isotypes other than IgM depends on the presence of IL-4 and/or IFN-γ, as well as IL-2 (1), the initial IL-2 that could be produced by naive helper T cells would be unlikely to be sufficient to drive an effective B cell response to antigen. Thus, it is likely that the development of an effector population studied here, is a prerequisite for a good B cell response to an antigen not previously encountered by the animal. The requirement for restimulation of the CD4$^+$ effectors makes sense in this context, because it would insure that they would not produce lymphokines until they encountered the appropriate antigen presented by B lymphocytes or other APC. Indeed, we have demonstrated that the population of lymph node cells, most enriched in effectors provides excellent help for a B cell response (Fig. 1 *B*).

The antigen-specific lymphokine producing T cells which develop in mice recently immunized with KLH are dependent upon, and possibly derived from a short-lived precursor population that is largely depleted by thymectomy (Fig. 4). The studies of Kappler et al. (27) have also shown that adult thymectomy prevents priming of helper activity in vivo. Adult thymectomy has been shown to deplete about 50% of CD4$^+$ T cells that are predominantly of naive phenotype (Pgp-1 high and CD45RB low), and enrich the reciprocal

population expressing a memory phenotype which, in contrast to the in vivo generated effector population, produces primarily IL-2 and only low levels of IL-3, IL-4, and IFN-γ (3, 28, and results discussed here). We have previously shown that CD4$^+$ effector cells which develop in vitro are derived from a short-lived or thymic dependent precursor population (3). In this study, we found that, as in the spleen, CD4$^+$ T cells that express high levels of Pgp-1 are enriched in the lymph nodes after adult thymectomy, correlating with the loss of the precursor population that is required for the generation of antigen-specific effector cells after primary immunization. Thus the effector cells we have generated in vivo and in vitro are analogous in this respect as well.

Antigen-specific effector cells generated in vivo are primarily low in expression of CD45RB and MEL-14 (Fig. 5 and 6), markers which also exhibit a reduced pattern of expression shortly after activation of T cells in vitro (16, 18). MEL-14 is a lymph node-specific homing receptor that is initially acquired by mature thymocytes just before their exit to the periphery (21). Recirculating naive T cells are thought to express MEL-14, allowing their extravasation to lymph nodes. MEL-14 is shed by T cells following activation by mitogens or allogeneic cells in vitro (16). The loss of surface MEL-14 correlates in vitro with changes in cell surface glycosylation of activated lymphocytes found in germinal centers, which are MEL-14$^-$ and unable to home to lymph nodes. B cells recently activated by antigen in vivo are also MEL-14$^-$ (29). If loss of surface MEL-14 is associated with antigen-activation in vivo, recirculation patterns of lymphocytes may be altered and at least temporarily permit localization in sites of antigen concentration during an immune response (30).

Changes in surface expression of CD45R that are induced by activation of CD4$^+$ T cells arise by alternative splicing of exons encoding the extracellular domain (31). The exon B-dependent isoforms of CD45R that are recognized by 23G2 are down-regulated on CD4$^+$ T cells following in vitro activation by allogeneic cells (18) as well as on memory CD4$^+$ helper T cells (17). In this study, we demonstrate that CD4$^+$ effector T cells with the capacity to secrete lymphokines shortly after in vivo immunization are predominately contained within the CD45RB low subset. These data are thus consistent with in vitro studies showing the loss of CD45R by CD4$^+$ T cells following antigen-stimulation (18). However, heterogeneity of CD4$^+$ effector cells is also detected on the basis of separation with CD45RB (Fig. 6 *B*). Although the CD45RB$^+$ effector cells are unable to produce IL-4 or IFN-γ, this subset can nonetheless secrete substantial titers of IL-2 and IL-3 in response to specific restimulation. In vitro studies of human lymphocytes have shown that changes in surface expression of CD45R occur over several days (31). Thus, it is not clear from this study whether the CD45RB$^+$ population of antigen-primed CD4$^+$ T cells represents a distinct subset of effectors, or a population in transition from CD45RB high-to-low expression. However, our data indicate that at the peak of antigen-specific effector development in vivo, transition to a memory surface phenotype has largely occurred, yet the effector population can be

clearly distinguished from resting memory CD4$^+$ T cells by the production of distinct arrays of lymphokines.

Our studies on the in vitro generation of CD4$^+$ effector cells have demonstrated that IL-4 and IFN-γ differentially regulate the development of CD4$^+$ subsets with discrete lymphokine-secreting phenotypes (22, 32), and other studies support the directive effects of IL-4 and IFN-γ in the generation of subsets of helper T cells (7, 8, 12). It is important to note that IL-2, the predominant lymphokine secreted by both resting naive and memory CD4$^+$ T cells in response to polyclonal activation, exerts no directive effect on the development of

CD4$^+$ subsets in vitro (22). Antigen-specific effector T cells present 3 to 8 d after immunization that reencounter antigen in vivo may potentially make large amounts of lymphokines. The lymphokines produced by these in vivo effectors are likely to play crucial roles not only in determining B cell responses, but also in directing the further development of subsets of CD4$^+$ effector T cells and perhaps memory cells. Further studies of parameters regulating the development of effector populations and the factors that determine the patterns of lymphokines they produce, may lead to the development of strategies for manipulating immune responses.

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