

# Interleukin 4 Production by CD4<sup>+</sup> T Cells from Allergic Individuals Is Modulated by Antigen Concentration and Antigen-presenting Cell Type

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## Summary

We have previously shown that CD4<sup>+</sup> T cells from allergic individuals are predisposed to produce interleukin (IL)-4 in response to allergens, and that allergen immunotherapy greatly reduced IL-4 production in an allergen-specific fashion. The mechanism that results in the reduction of IL-4 synthesis in treated individuals is unknown, but because clinical improvement during immunotherapy is associated with the administration of the highest doses of allergen, we hypothesized that high concentration of allergen results in the downregulation of IL-4 synthesis in CD4<sup>+</sup> T cells. In this report, we demonstrated that CD4<sup>+</sup> T cells from allergic donors produced high levels of IL-4 when stimulated with low concentrations of allergen (0.003–0.01  $\mu\text{g/ml}$ ), particularly when B cell-enriched populations presented the antigen. In contrast, the same responding CD4<sup>+</sup> T cell population produced little IL-4 when stimulated with high concentrations of allergen (10–30  $\mu\text{g/ml}$ ), especially when monocytes were used as antigen-presenting cells (APC). The quantity of IL-4 produced was also found to be inversely related to the extent of proliferation of the CD4<sup>+</sup> T cells in response to allergen/antigen; maximal proliferation of CD4<sup>+</sup> T cells occurred in response to high concentrations of antigen when IL-4 production was minimal. Antigen presentation by B cell-enriched populations, instead of monocytes, induced less CD4<sup>+</sup> T cell proliferation, but induced much greater IL-4 synthesis. Moreover, the addition of increasing numbers of APC (either B cells or monocytes) to cultures containing a constant number of responder T cells resulted in increased T cell proliferation and decreased IL-4 production. These results indicate that the circumstances under which memory T cells are activated, as well as the strength of the proliferative signal to T cells, greatly affect the quantity of IL-4 produced. Thus, our observations that the cytokine profile of allergen-specific memory CD4<sup>+</sup> T cells can indeed be modulated by the antigen dose and APC type suggest that methods that preferentially enhance allergen uptake by monocytes and that enhance T cell proliferation will improve the clinical efficacy of immunotherapy in the treatment of allergic disease.

The precise mechanisms by which allergen immunotherapy achieves clinical improvement in patients with allergic rhinitis is poorly understood. Several studies have demonstrated that immunotherapy induces a rise in IgG blocking antibodies, particularly IgG4 (1, 2), whereas other studies have demonstrated that CD8<sup>+</sup> suppressor cells are generated during immunotherapy, which can modulate allergen-specific IgE production (3). However, these effects are not always observed in patients on immunotherapy, which suggests that other mechanisms are operating. Since natural exposure to allergen occurs generally by inhalation, whereas treatment with immunotherapy involves subcutaneous administration of allergen, it has been postulated that the route of antigen exposure may significantly affect the cytokine profiles that develop in antigen/allergen-specific T cells (4). In addition, since the amount of allergen administered during immunotherapy is much greater than the amount inhaled,

we and others hypothesized that higher antigen concentrations would reduce IL-4 synthesis in allergen-specific CD4<sup>+</sup> T cells, possibly by altering the type of antigen-presenting cells or monokines involved (5–8).

We have previously demonstrated that allergen immunotherapy, which is a clinically effective treatment for patients with allergic rhinitis and which involves the subcutaneous administration of increasing doses of allergen, greatly reduces the production of IL-4 in allergen-specific CD4<sup>+</sup> T cells. Allergen immunotherapy decreases IL-4 production by allergen-specific CD4<sup>+</sup> T cells to levels observed with T cells from nonallergic subjects, or to levels induced by nonallergenic antigens, such as tetanus toxoid (9). Inasmuch as the development of allergic disease with increased IgE synthesis is thought to be caused by excessive production of IL-4 in allergen-specific CD4<sup>+</sup> T cells (10–15), allergen immunotherapy, by reducing IL-4 synthesis and altering the cytokine profiles of allergen-

specific T cells, brings about clinical improvement in allergic individuals by affecting the underlying regulatory T cells that control the disease.

The purpose of our current study was to investigate a possible mechanism by which immunotherapy causes a reduction in the quantity of IL-4 produced by allergen-specific CD4+ T cells. Since clinical improvement during immunotherapy is associated with the administration of the highest doses of allergen (16), we hypothesized that IL-4 synthesis in CD4+ T cells is downregulated by high concentrations of allergen and/or when antigen is presented by monocytes rather than by B cells. We, thus, demonstrate that the cytokine profile of peripheral blood CD4+ T cells taken from allergic individuals can, in fact, be altered by antigen dose and antigen-presenting cell type in vitro, and we propose that this is a significant mechanism responsible for the success of allergen immunotherapy.

## Materials and Methods

**Donors.** Allergic donors were characterized by positive immediate skin test reactivity to dust mite antigen, *Dermatophagoides farinae* (*Der.p.*)<sup>1</sup>, and/or to rye grass pollen allergen, *Lolium perenne* (*Lol.p.*), and by serum IgE levels  $\geq 170$  IU/ml. Nonallergic subjects did not exhibit immediate skin test reactivity to these allergens and had total serum IgE levels  $< 50$  IU/ml. All donors gave informed consent, and experimental protocols were approved by the Stanford University Administrative Panel on Human Subjects in Medical Research.

**Antigens.** Purified *Lol.p.* group I antigen (National Institute of Allergy and Infectious Diseases, Rockville, MD) was used at final concentrations ranging from 1 ng/ml to 50  $\mu$ g/ml. Partially purified house dust mit (*Der.p.*) antigen preparation was generously provided by S. Dreborg (Pharmacia, Uppsala, Sweden) at  $10^5$  biological equivalent/ml and was used at final concentrations ranging from 20 ng/ml to 20  $\mu$ g/ml in culture. Tetanus toxoid (TT) antigen (Connaught Laboratories, Inc.; Swiftwater, PA) was used in the range of 3 ng/ml to 30  $\mu$ g/ml in culture.

**Isolation of CD4+ T Cells.** Peripheral blood T cells were obtained by rosetting with 2-aminoethylisothiuronium bromide-SRBC (17) followed by depletion of SRBC with lysing solution (0.155 M  $\text{NH}_4\text{Cl}$ , 10 mM  $\text{KCO}_3$ ). CD8+ cells were removed from the preparation by treatment with anti-OKT8 (anti-CD8) mAb followed by the addition of rabbit complement (Pel-Freez Biologicals; Rogers, AR), as previously described (18). Cells treated in this manner were  $\leq 6\%$  CD8 positive and  $\geq 92\%$  CD4 positive.

**Preparation of Antigen-presenting Cells.** PBMC were depleted of T cells by rosetting with aminoethylisothiuronium bromide-treated SRBC as described above. The remaining B cells and monocytes (E-rosette negative fraction [ $\text{E}^-$ ]) were washed three times in PBS, treated with mitomycin C (50  $\mu$ g/ml; Sigma Chemical Co., St. Louis, MO) for 45 min at 37°C, and washed extensively before culture. In other experiments, E-negative cells were irradiated (2,500 rad) before culture. To purify monocytes/macrophages, PBMC ( $5-8 \times 10^6$ /ml) were incubated in 60 mm plastic dishes in complete medium for 1 h at 37°C. Nonadherent cells were removed

by gently washing the dishes with warm RPMI 1630 media containing 5% FCS. The remaining adherent cells were harvested with cold PBS, washed three times, and irradiated at 2,500 rad before culture. Monocytes isolated in this manner were typically  $>99\%$  OKM1+ CD14<sup>hi</sup> CD33+. Purified B cells were obtained by removing monocytes from the  $\text{E}^-$  fraction with two rounds of adherence to plastic, as described above, and by passage of the cells over Sephadex G10 (19). The remaining cells were then incubated with anti-OKM1 (anti-CD1; Ortho Diagnostic Systems, Raritan, NJ) mAb for 45 min at 4°C, followed by the addition of rabbit complement (1:3 [vol:vol] Pel-Freez Biologicals), and placed at 37°C for 30 min. After washing three times with RPMI 1630 containing 5% FCS, the B lymphocyte-enriched population was incubated with 50  $\mu$ g/ml mitomycin C for 45 min at 37°C and washed extensively before culture. The B lymphocyte-enriched population isolated in this manner was typically  $< 3\%$  OKM1+ and 6–11% CD14<sup>lo</sup> CD33+ (which identify precursors of dendritic cells).

**Proliferation Assay.** PBMC were isolated from heparinized blood by flotation over Ficoll-Hypaque (Sigma Chemical Co.), as previously described (18). PBMC ( $2 \times 10^5$ /well) were cultured in triplicate in 96-well plates in media alone (DMEM containing 10% AB+ serum [Hazleton Biologics, Inc., Lenexa, KS], vitamins, 1 mM sodium pyruvate, 5  $\mu$ g/ml gentamycin, and 2 mM l-glutamine) or with antigen at the appropriate concentration. After 5 d, cultures were pulsed with [<sup>3</sup>H]thymidine and harvested 18 h later. Incorporation of [<sup>3</sup>H]thymidine (cpm) was then determined by scintillation counting. Where indicated in the text, proliferation of purified CD4+ T cells ( $1.5 \times 10^5$ /well), in response to antigen, was assessed in a similar manner using either irradiated monocytes or mitomycin C-treated E-negative cells ( $5 \times 10^4$ /well) as APC.

**Cell Cultures.** Purified CD4+ T cells and APC were cultured in 24-well plastic dishes (Nunc, Naperville, IL) at  $2 \times 10^6$  cells/ml in 10%  $\text{CO}_2$  with the appropriate antigen: TT, dust mite antigen, or rye grass pollen antigen, as previously described (9). After 6 d of culture, cells were washed once in PBS and recultured with fresh APC, fresh antigen, and 10 U/ml human recombinant (hr)IL-2 (Amgen Biologicals, Thousand Oaks, CA). Between days 6 and 12, cultures were expanded as necessary. At day 12 of culture, cells were washed three times in PBS and resuspended in complete medium at  $2 \times 10^6$  cells/ml with PHA-P (1:200 [vol:vol]; Difco Laboratories Inc., Detroit, MI) and 1 ng/ml of PMA. Supernatants were collected after 24 h and kept at  $-80^\circ\text{C}$  until they were assayed for cytokine content. Where indicated, cells were processed for RNA isolation. In some experiments, antigen specificity of cultures was confirmed by isolating blast cells at day 6 of culture via centrifugation through a discontinuous Percoll gradient at 1,500 rpm for 45 min followed by culture of those cells at the 27–45% Percoll interface for an additional 6 d of culture. Similar results were obtained using both culture protocols.

**Quantitation of Cytokines.** hIL-2 was measured by biological assay, using the murine indicator HT-2 cell line (generously provided by Dr. Sam Strober, Stanford), as previously described (18). hIL-4 was quantified by ELISA as previously described (10).

**RNA Isolation and Northern Blot Analysis.** Analysis of mRNA for IL-4 was carried out with RNA extracts from CD4+ T cells that had been cultured with various concentrations of allergen and mitomycin C-treated E-cells as APC, for 12 d, as described above. After a final 8 h stimulation with PHA+PMA, RNA was extracted from cells by a rapid isolation procedure (20), fractionated on formaldehyde-agarose gels, and transferred and cross-linked to nylon membranes (Hybond-N; Amersham Corp., Arlington Heights, IL). The cDNA probe for IL-4 was a 0.7-kb BamHI digest out of pcD 3123b vector (21) (generously provided by Dr. Jan de Vries, DNAX,

<sup>1</sup> Abbreviations used in this paper: *Der.p.*, *Dermatophagoides farinae*;  $\text{E}^-$ , erythrocyte rosette-negative; hr, human recombinant; *Lol.p.*, *Lolium perenne*; TT, tetanus toxoid.

Palo Alto, CA). The probe was labeled using random hexanucleotide primers (Pharmacia) with  $\alpha$ - $^{32}$ P-dATP (Amersham). Membranes were hybridized at 42°C for 16 h (overnight) with labeled cDNA probe in Northern cocktail containing 50% formamide, 1 M NaCl, 1×PE, 10% dextran sulfate, and 100  $\mu$ g/ml salmon sperm DNA. Blots were washed twice at room temperature for 15 min each with 0.2 × SSC/0.1% SDS (1×SSC = 0.15 M NaCl, 0.17 M sodium citrate) followed by two more washes at 56°C for 30 min each with 0.2 × SSC/0.1% SDS. Blots were exposed at -70°C to XAR film (Eastman Kodak Co., Rochester, NY). Equal loading of RNA was assessed by ethidium bromide staining of the gel. After exposure, blots were stripped with 0.1% SDS at 100°C before hybridizing with a second probe.

## Results

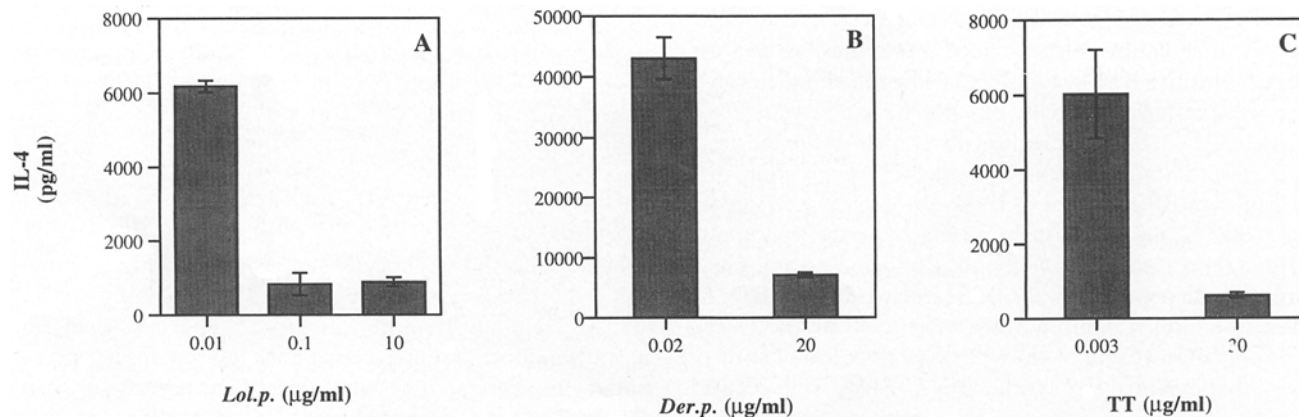
**Induction of IL-4 Production in CD4+ T Cells Is Maximal at Low Doses of Antigen.** Peripheral blood CD4+ T cells from allergic donors were cultured with allergen (*Lol.p.*) and mitomycin C-treated E-negative cells (containing B lymphocytes and monocytes) as APC, and the cytokine profile of the responding cells was then determined (10). As depicted in Fig. 1 A, maximal IL-4 synthesis by CD4+ T cells was observed at low concentrations of allergen (0.01  $\mu$ g/ml) while significantly less IL-4 synthesis was seen at higher concentrations of allergen (0.1 and 10  $\mu$ g/ml). Although greater quantities of IFN- $\gamma$  were produced at higher, as compared to lower, concentrations of antigen, the differences were not statistically significant (data not shown). IL-2 synthesis varied between experiments, but overall it was not significantly modulated by antigen concentration. This pattern of IL-4, IL-2, and IFN- $\gamma$  synthesis was consistently observed in more than ten experiments.

We next examined whether the dosage of other allergens and/or nonallergens was able to modulate CD4+ T cell cytokine synthesis in a similar manner. As seen in Fig. 1 B, stimulation of purified CD4+ T cells from an allergic donor with low and high concentrations of *Der.p.* elicited responses

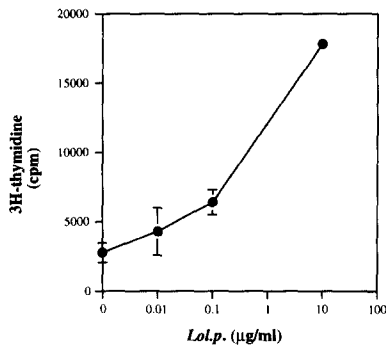
similar to those found with high and low concentrations of *Lol.p.* with much enhanced IL-4 synthesis at the lower concentration of allergen. Although the absolute amount of IL-4 produced varied with each donor, the relative difference in IL-4 synthesis at low and high concentrations of allergen was consistent in each experiment. In addition, as demonstrated in Fig. 1 C, stimulation of CD4+ T cells isolated from an allergic subject with low and high concentrations of TT also revealed maximal IL-4 synthesis at low antigen concentrations. IL-4 production at low antigen concentrations was obtained only in subjects recently boosted with TT, thus maximizing the number of TT-specific B cells in the responding population and suggesting that antigen-specific B cells, rather than nonantigen-specific B cells, were responsible for antigen presentation. Comparable results were observed in response to TT in a recently boosted nonallergic donor (data not shown), demonstrating a consistent effect with all three antigens tested.

Since the degree of proliferation of CD4+ T cells is affected by the antigen concentration, we examined the proliferative response to CD4+ T cells from allergic individuals in the first 6 d of culture and determined how it changed as a function of allergen concentration. Fig. 2 shows that maximal proliferation of the CD4+ T cells occurred at the highest concentrations of allergen (10  $\mu$ g/ml), and that, at very low concentrations of allergen, only minimal T cell proliferation was observed. Similar results were observed when *Der.p.* or TT were used as the antigen (data not shown). Thus, the quantity of IL-4 produced by allergen/antigen-specific CD4+ T cells, which was maximal with low concentrations of antigen, was inversely related to the amount of T cell proliferation as measured during the first week of stimulation.

The reduced production of IL-4 at higher concentrations of antigen (Fig. 1) could not be explained by increased consumption of IL-4 in cultures with increased proliferation, since the supernatants for assay were generated over a short period of time (24 h) and since Northern analysis of the T cells for



**Figure 1.** IL-4 production by CD4+ T cells is enhanced at low antigen concentrations. Purified CD4+ T cells were cultured with mitomycin C-treated E- cells (B cells and monocytes) as APC and stimulated with the indicated concentrations of allergen: (A) *Lol.p.*, (B) *Der.p.*; or (C) nonallergen, TT. Cytokine production was assessed from supernatants generated after 12 d of culture (see Materials and Methods) and is expressed as the mean  $\pm$  SD of triplicate cultures. These results are representative of 10 experiments with allergen and two experiments with TT.

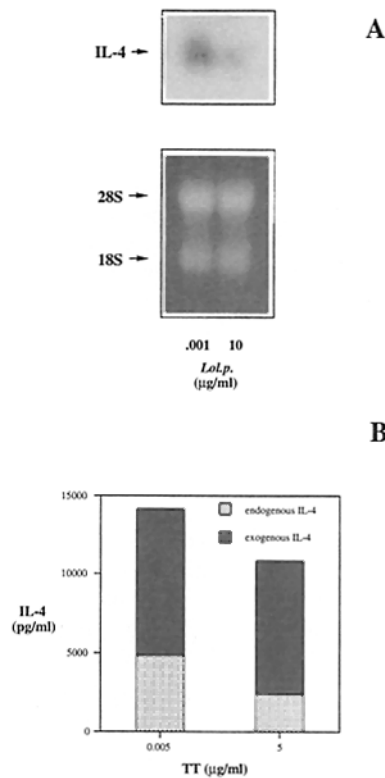


**Figure 2.** CD4<sup>+</sup> T cell proliferation is maximal at high allergen/antigen concentrations. Purified CD4<sup>+</sup> T cells were cultured with mitomycin C-treated E<sup>-</sup> cells as APC with the indicated concentrations of allergen. After 5 d, the cultures were pulsed with [<sup>3</sup>H]thymidine and harvested 18 h later. Results are expressed as incorporation of [<sup>3</sup>H]thymidine (cpm) ± SD of triplicate wells. These results are representative of eight experiments.

IL-4 transcripts (Fig. 3 A) demonstrated that the quantity of mRNA for IL-4 varied directly with the quantity of IL-4 protein found in supernatants. In addition, mock experiments performed by adding known quantities of IL-4 at the time the supernatants were generated showed that the T cells generated at high versus low antigen concentrations did not use IL-4 differentially (Fig. 3 B).

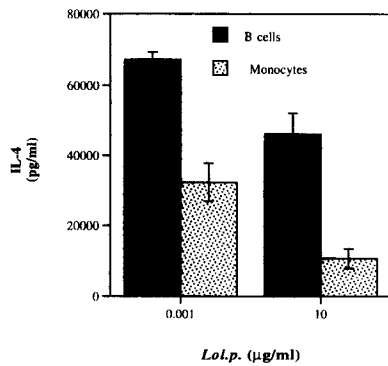
**Antigen Presentation by B Cell-enriched Populations Favors IL-4 Synthesis by CD4<sup>+</sup> T Cells.** A number of investigators have demonstrated that, particularly when low concentrations of antigen are present, antigen-specific B cells are 1,000- to 10,000-fold more efficient than macrophages in presenting antigen to T cells, mainly because B cells bind antigen (through Ig receptors) much more effectively than macrophages (22-24). Since we observed that at low concentrations of antigen more IL-4 was induced, we reasoned that antigen presentation by B cells occurred preferentially at low antigen concentrations, favoring IL-4 synthesis, while antigen presentation by monocytes occurred preferentially at high antigen concentrations, resulting in decreased IL-4 synthesis. To examine this possibility, we compared the cytokines induced in CD4<sup>+</sup> T cells by irradiated versus mitomycin C-treated E<sup>-</sup> cells. Inasmuch as the E<sup>-</sup> population includes both monocytes, as well as radiation-sensitive B cells, and since irradiation eliminates antigen presentation by B cells, but not by monocytes (25), the irradiated population functionally contained monocytes, not B cells. The mitomycin C-treated E<sup>-</sup> population induced substantial quantities of IL-4. However, irradiation of the E<sup>-</sup> cells (2,500 rad) resulted in markedly reduced levels of IL-4, a decrease from 16,585 pg/ml to 3,882 pg/ml. Furthermore, both populations of APC (irradiated and mitomycin C-treated E<sup>-</sup> cells) induced comparable levels of CD4<sup>+</sup> T cell proliferation (data not shown). Therefore, these results suggest that maximal IL-4 synthesis by CD4<sup>+</sup> T cells occurs when B cells, rather than monocytes, present antigen to allergen-specific T cells.

To more directly compare the ability of equal numbers of B lymphocytes and monocytes to induce CD4<sup>+</sup> IL-4 synthesis, E<sup>-</sup> cells from an allergic donor were separated into



**Figure 3.** Decreased IL-4 synthesis at high antigen concentrations is not due to enhanced uptake. (A): IL-4 mRNA expression correlates with quantity of IL-4 protein in supernatants. Purified CD4<sup>+</sup> T cells were cultured with mitomycin C-treated E<sup>-</sup> cells as APC (as described in Fig. 1) with Lol.p., 0.001 µg/ml (lane 1), or 10 µg/ml (lane 2). At day 12, cells were stimulated with mitogen for 6 h and RNA isolated for Northern blot analysis using a cDNA probe specific for human IL-4. Ethidium bromide staining of 18S and 28S RNA is presented as a demonstration of equal loading of RNA. The exposure time was 3 d. Levels of IL-4 protein in supernatants of parallel cultures were 25,387 pg/ml at 0.001 µg/ml Lol.p. and 3,410 pg/ml at 10 µg/ml Lol.p. (B): Differences in IL-4 production at high and low antigen concentrations are not due to differential IL-4 uptake. Purified CD4<sup>+</sup> T cells were cultured with mitomycin C-treated B lymphocyte-enriched cells as APC, as described in Fig. 1, with the indicated concentrations of TT. At day 12, cells were washed, resuspended at 2 × 10<sup>6</sup> cells/ml, and restimulated with PHA + PMA. In some cultures, IL-4 (10,000 pg) was added at the time of restimulation to determine if the restimulated T cells differentially used IL-4. IL-4 levels, without the addition of IL-4, was, for 0.005 µg/ml TT; 5,020 pg/ml, and for 5 µg/ml TT; 2,111 pg/ml indicating that 9,280 and 8,530 pg/ml, respectively, of the added IL-4 remained in the cultures. A control well containing 10,000 pg/ml IL-4 in the absence of cells was assayed as containing 10,800 pg/ml after 24-h culture.

populations of B lymphocytes and monocytes. Fig. 4 shows that a cell population highly enriched with B lymphocytes induced significantly more IL-4 in CD4<sup>+</sup> T cells than did an equal number of monocytes at both low and high concentrations of allergen. The monocytes induced less IL-4 than did the B cell-enriched population, whether the monocytes were treated with mitomycin C or with irradiation (data not shown). In most experiments, monocytes induced more IFN-γ production by CD4<sup>+</sup> T cells than did the B cell population (data not shown). Thus, antigen presentation by B lympho-



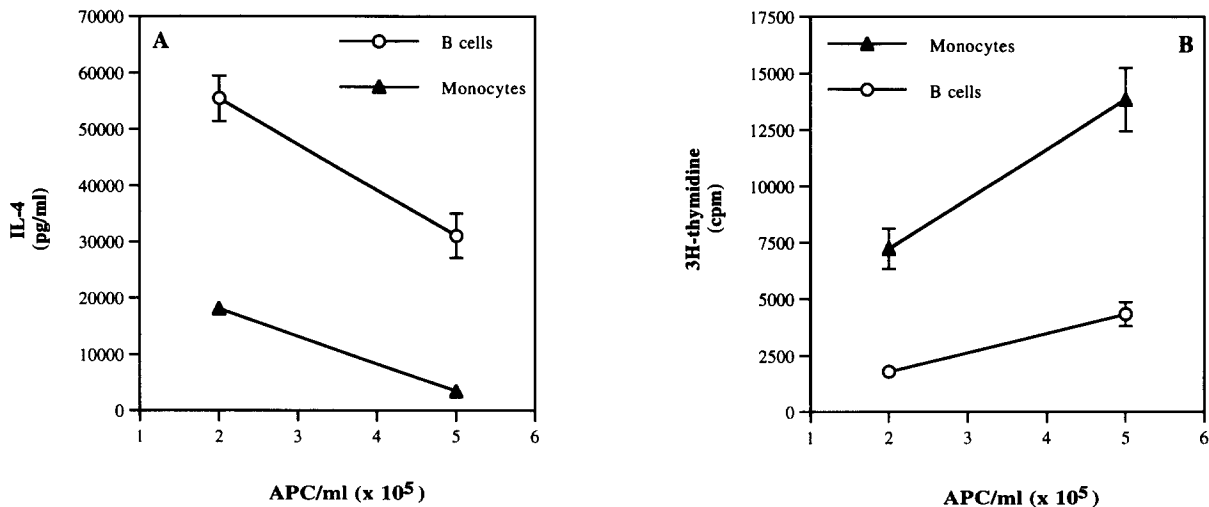
**Figure 4.** Purified B cells as APC are more effective than monocytes at inducing IL-4 synthesis in CD4+ T cells. Purified CD4+ T cells were cultured with mitomycin C-treated B cells or monocytes, as described in Fig. 1, with the indicated concentrations of allergen. Cytokine levels were determined as in Fig. 1 and are expressed as the mean  $\pm$  SD of triplicate cultures. These results are representative of five experiments.

cyte-enriched populations was superior to that of monocytes for the induction of IL-4 synthesis in memory CD4+ T cells.

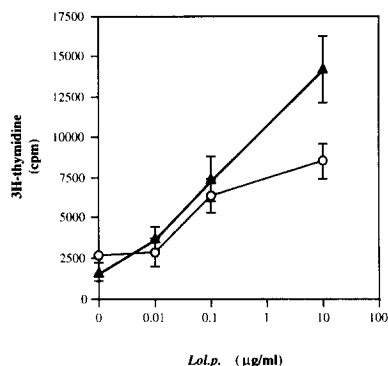
**Increasing the APC: CD4+ T Cell Ratio Inhibits IL-4 Synthesis.** Inasmuch as enhanced IL-4 synthesis was observed at lower concentrations of antigen, even when using monocytes as APC, we asked if the number of APC in culture and, consequently, the strength of the signal delivered to the CD4+ T cell also affected lymphokine synthesis. Therefore, we examined the effect of increasing the ratio of APC to CD4+ T cells in culture on IL-4 synthesis. As demonstrated in Fig. 5 A, increasing the number of APC in culture while keeping the number of responder T cells constant resulted in decreased IL-4 synthesis using both types of APC, although the B cell-enriched population was far better than mono-

cytes in inducing IL-4 synthesis at all APC concentrations tested. Moreover, decreased IL-4 synthesis at higher concentrations of APC correlated with increased CD4+ T cell proliferation (Fig. 5 B), although monocytes induced greater CD4+ T cell proliferation at both concentrations of APC. Fig. 6 shows that, with both types of APC, CD4+ T cell proliferation was also enhanced with increasing concentrations of allergen, with monocytes inducing greater proliferation, particularly at higher antigen concentrations. These results again indicate that the quantity of IL-4 produced by T cells is inversely related to the amount of T cell proliferation, that stronger signaling from APC (with increased antigen or increased APC number) is associated with reduced IL-4 synthesis, and that B cells are superior to monocytes at inducing IL-4 synthesis in memory CD4+ T cells.

**CD4+ Lymphokine Synthesis Is Determined by the APC Present at the Primary Stimulation.** Since induction of maximal IL-4 synthesis in our in vitro cultures required two stimulations with antigen and APC, we asked whether the presence of B lymphocyte-enriched cells as the APC type was required for both the first and second stimulations. CD4+ T lymphocytes were cultured with allergen and either B lymphocyte-enriched cells or monocytes for 6 d, at which time cells were restimulated with allergen, hrIL-2, and either the original or the other APC type for the second stimulation. As depicted in Fig. 7, maximal CD4+ IL-4 synthesis occurred using B lymphocyte-enriched cells as APC during the first stimulation, whether or not the B cell population was used during the second stimulation. In contrast, use of monocytes as APC for the first stimulation resulted in the induction of low levels of IL-4. Thus, maximal IL-4 synthesis by CD4+ memory T lymphocytes occurred when a B lymphocyte-enriched population was used as APC during the first stimulation, indicating that circumstances with which the resting



**Figure 5.** Increasing the APC: CD4+ T cell ratio inhibits IL-4 synthesis, yet enhances CD4+ T cell proliferation. A fixed number of purified CD4+ T cells ( $1.5 \times 10^6$ /ml) were cultured with either mitomycin C-treated B cells (open circles) or monocytes (closed triangles) at the indicated concentrations with  $0.001 \mu\text{g/ml}$  Lol.p. Cytokine levels were determined, as described in Fig. 1, and are expressed as the mean  $\pm$  SD of triplicate cultures (A). CD4+ T cell proliferation was determined as described in Fig. 2, and results are expressed as incorporation of [ $^3\text{H}$ ]thymidine  $\pm$  SD of triplicate wells (B). These results are representative of three experiments.



**Figure 6.** Both monocytes and purified B cells induce augmented CD4+ T cell proliferation with increasing concentrations of allergen. Proliferative response of CD4+ T cells ( $1.5 \times 10^6$ /ml) to various concentrations of allergen presented by mitomycin C-treated B cells (open circles;  $5 \times 10^5$ /ml) or monocytes (closed triangles;  $5 \times 10^5$ /ml). Results are expressed as the incorporation of [ $^3$ H]thymidine  $\pm$  SD of triplicate cultures over the last 18 h of a six day culture period. These results are representative of four experiments.  $\blacktriangle$ , monocytes;  $\circ$ , B cells.

memory T cell was activated determined whether IL-4 was produced, and that manipulation of the cytokine profile of activated memory T cells at the time of the second stimulation was not possible.

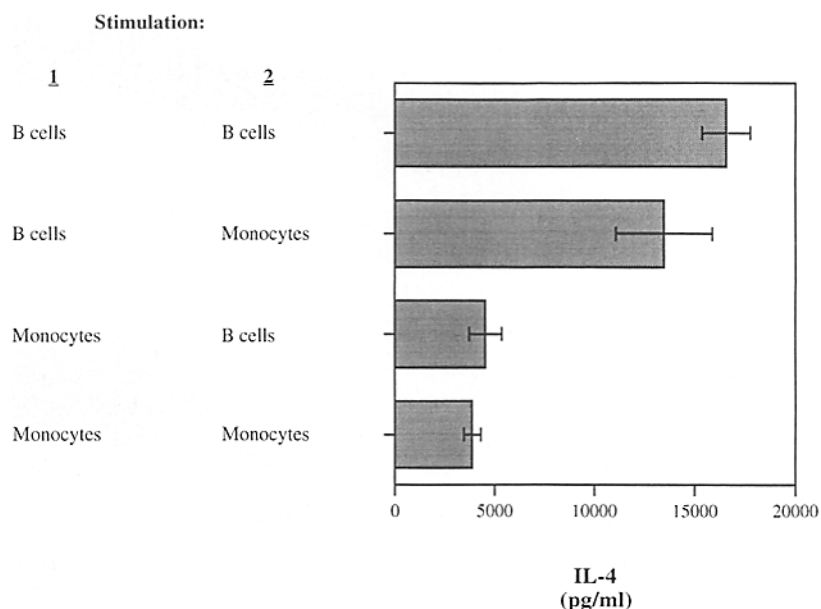
## Discussion

In the current report, we demonstrated, using human memory CD4+ T cells, that cytokine production can be modulated by the antigen dose and APC type and proposed that allergen immunotherapy operates through such mechanisms. Stimulation of CD4+ T cells from allergic individuals with low concentrations of allergen or antigen (1–100 ng/ml) in vitro, which enhances antigen presentation by B cells (22–24),

resulted in enhanced IL-4 synthesis, whereas stimulation of the same CD4+ T cell population with high concentrations of allergen or antigen (10–30  $\mu$ g/ml) resulted in minimal IL-4 synthesis. Moreover, we demonstrated that B lymphocyte-enriched populations were more effective than monocytes at inducing IL-4 synthesis in allergen-specific CD4+ T cells and that the quantity of IL-4 produced was inversely related to the extent of proliferation of the CD4+ T cells. It is possible that, at low antigen concentrations, contaminating Fc $\epsilon$ RI+ dendritic cells, expressing antigen-specific IgE, presented antigen instead of B cells and induced high levels of IL-4 (26). However, this is unlikely since irradiation, which does not affect antigen presentation by dendritic cells (27) but eliminates antigen presentation by B cells, greatly reduced the capacity of the B cell-enriched population to induce IL-4 production.

Previous studies examining the regulation of cytokine synthesis in CD4+ T cells have demonstrated that cytokine production in naive CD4+ T cells is relatively easy to manipulate by controlling the presence of IL-4, or by the addition of anti-IL-4 mAb or anti-IFN- $\gamma$  mAb to the microenvironment of the responding T cells (28, 29). However, the cytokine profiles of memory CD4+ T cells in established immune responses have been more difficult to manipulate, and attempts to alter the cytokine profile of CD4+ T cells with cytokines or anticytokine mAb have been largely unsuccessful (30–32). Although the presence of IL-12 can enhance IFN- $\gamma$  synthesis, a recent report has demonstrated that IL-12 does not reduce IL-4 synthesis in human CD4+ memory T cells (33). However, we have previously shown that allergen immunotherapy can significantly reduce the quantity of IL-4 produced by allergen-specific CD4+ T cells (9), and our current results indicate that immunotherapy may control the cytokine profiles of allergen-specific memory CD4+ T cells by affecting the antigen concentration and the APC type.

The idea that antigen presentation by B cells enhances IL-4



**Figure 7.** CD4+ T cell lymphokine synthesis is determined by the APC present at the initial stimulation in vitro. Purified CD4+ T cells were cultured with 50  $\mu$ g/ml *Lol.p* and either mitomycin C-treated B cells or irradiated macrophages for 6 d, at which time cells were restimulated with allergen, rhIL-2, and either the original or a different APC for the second stimulation. At day 12, cytokine levels were determined and are expressed as the mean  $\pm$  SD of triplicate cultures. These results are representative of three experiments.

synthesis in memory CD4<sup>+</sup> T cells has not been studied extensively in the past. Our results demonstrating that IL-4 synthesis is enhanced in human memory CD4<sup>+</sup> T cells when antigen is presented by B lymphocyte-enriched cells are similar to those results with murine CD4<sup>+</sup> T cells in which we demonstrated that KLH-primed murine CD4<sup>+</sup> T cells synthesized more IL-4 and less IFN- $\gamma$  in vitro when B lymphocytes, and not macrophages, presented antigen at low concentrations (10 ng/ml). However, in that study the quantity of IL-4 induced by both B cells and macrophages was equivalent at higher concentrations of antigen (1  $\mu$ g/ml) (5). Antigen presentation by B cells has also been shown to preferentially stimulate established Th2 rather than Th1 clones (34), but since T cell clones have stable cytokine profiles, the type of APC used did not affect the cytokine profile of the responding T cell. In contrast, because resting memory CD4<sup>+</sup> T cells must differentiate upon stimulation with antigen before secreting IL-4 (5, 10), we believe that, in our experimental system, antigen presentation by B lymphocytes actually modulated the cytokine profile of the responding T cells and did not simply select out T cells already committed to produce high levels of IL-4. However, once the resting memory CD4<sup>+</sup> T cells were activated in vitro by a specific APC type, the cytokine profile of the activated T cell became relatively fixed and resistant to subsequent change (Fig. 7). Future studies, though, that examine memory T cells at the single cell level will be required to directly demonstrate the plasticity in the cytokine profiles of individual resting memory CD4<sup>+</sup> T cells.

Since B cells are extremely efficient at presenting antigen when the antigen concentration is low (22, 23), our studies showing that antigen presentation by B lymphocyte-enriched cells preferentially induce IL-4 are consistent with previous in vivo and in vitro observations that IgE synthesis (and, therefore, IL-4 synthesis) is optimal at low antigen doses (35, 36). We demonstrated that the induction of substantial quantities of IL-4 in T cells by B lymphocyte-enriched cells, particularly at low antigen concentrations, occurred with minimal CD4<sup>+</sup> T cell proliferation. In contrast, induction by monocytes of high levels of IFN- $\gamma$  synthesis and low levels of IL-4 synthesis was associated with vigorous T cell proliferation, particularly at high concentrations of antigen. These observations suggest that the induction of Th2 cytokines in human CD4<sup>+</sup> T cells does not require substantial T cell proliferation, whereas the induction of Th1 cytokines requires active proliferation.

The dissociation of T cell proliferation and IL-4 production has been observed in the past in several murine systems. First, Evavold et al., using hemoglobin-specific murine T cell clones, showed that alteration of the amino acid sequence of the relevant hemoglobin peptide, which causes a reduction in its affinity for the hemoglobin-specific T cell receptor, results in decreased T cell proliferation, but not in decreased IL-4 production, by the clone (37). That IL-4 secretion does not require significant T cell proliferation is also supported by studies with the antigen collagen type IV whose peptides have low affinity for non-H-2<sup>S</sup> MHC molecules and induce poor CD4<sup>+</sup> T cell proliferation, yet substantial IL-4 produc-

tion in non-H-2<sup>S</sup> mice. In contrast, the same peptides bind effectively to H-2<sup>S</sup> MHC molecules and induce vigorous T cell proliferation with the production of IL-2 and IFN- $\gamma$  in H-2<sup>S</sup> mice (38). Finally, studies by Gajewski et al. show that the development of Th2, but not Th1 cells, from Th0 cells is enhanced by the relative lack of costimulatory signaling from APC (39). Although their study suggests that IL-4 synthesis correlates with the induction of anergy in T cells, our studies indicate that IL-4 synthesis can occur without the development of anergy in the T cells, since our IL-4-producing cells could proliferate when restimulated with antigen and APC (data not shown).

Taken together, these results suggest that the induction of IL-4 synthesis involves T cell signaling mechanisms that are distinct from those required for IL-2 and IFN- $\gamma$  synthesis, and that stronger T cell activation signals in memory CD4<sup>+</sup> T cells (i.e., with greater TCR cross-linkage, higher antigen concentration, or higher avidity of antigen peptide for MHC class II or TCR) induce greater T cell proliferation and less Th2 cytokines. These differences in signaling may subsequently result in the development of effector CD4<sup>+</sup> T cells with alternate activation pathways; one using signaling by the hydrolysis of phosphatidylinositol biphosphate and activation of protein kinase C (Th1 pathway), and the other relying on a phosphatidylinositol biphosphate- and protein kinase C-independent pathway (Th2 pathway) (40, 41). In addition, it is possible that very potent signaling to T cells (with ultra high levels of antigen or antigen peptide, for example) results in T cell anergy or apoptosis (42, 43), perhaps analogous to the situation observed in thymic differentiation during which high concentrations of antigen result in negative selection (44). In the peripheral lymphoid organs in vivo, very high concentrations of antigen may also eliminate T cells bearing high affinity receptors for antigen but leave T cells bearing low affinity receptors. On subsequent exposure to antigen, such T cells may proliferate poorly to antigen and produce Th2 cytokines as is seen in mice after treatment with very high concentrations of an aqueous protein antigen (45). Alternatively, the cytokine profiles of T cells may be controlled by APC-secreted cytokines, such as IL-12, which greatly enhances IFN- $\gamma$  synthesis (33, 46, 47), or IL-10, which inhibits IFN- $\gamma$  production in T cells (48). In our cultures, however, IL-12 and IL-10 had only minimal effects on the cytokine profiles of CD4<sup>+</sup> T cells, since the addition of a neutralizing anti-IL-12 mAb did not affect cytokine synthesis and since the addition of a neutralizing anti-IL-10 mAb decreased IL-4 synthesis only slightly (data not shown).

In summary, our results demonstrate a mechanism by which allergen immunotherapy reduces IL-4 synthesis in allergen-specific memory CD4<sup>+</sup> T cells. We showed that the quantity of IL-4 produced by CD4<sup>+</sup> T cells can be modified by antigen concentration and APC type. These results suggest that modifications of immunotherapeutic methods that enhance allergen uptake and presentation by monocytes and that enhance allergen-specific T cell proliferation will greatly improve the clinical efficacy of immunotherapy in the treatment of allergic disease. Finally, our observations suggest that im-

munotherapy, with high antigen doses that induce vigorous T cell proliferation, may be applicable to other diseases in which the production of inappropriately high levels of IL-4

in memory CD4<sup>+</sup> T cells requires reduction in an antigen-specific manner (e.g., lepromatous leprosy and other infections).

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