

Allergens, IgE, mediators, inflammatory mechanisms

The relative contribution of IL-4 and IL-13 to human IgE synthesis induced by activated CD4⁺ or CD8⁺ T cells

Juha Punnonen, MD, PhD, Hans Yssel, PhD, and Jan E. de Vries, PhD

Palo Alto, Calif.

The relative contribution of IL-4 and IL-13 to the regulation of IgE synthesis has remained relatively poorly characterized, partially because of lack of suitable animal models. We have studied the roles of IL-4 and IL-13 in human IgE synthesis induced by supernatants derived from activated CD4⁺ or CD8⁺ T cell clones. Neutralizing anti-IL-4 and anti-IL-13 monoclonal antibodies (mAbs) inhibited IgE synthesis induced by anti-CD40 mAbs and supernatants from CD4⁺ T cells by an average 61% and 42%, respectively ($n = 25$). Recombinant IL-13 had additive effects on IL-4-induced IgE synthesis, but only when IL-4 was present at low concentrations. Accordingly, IL-4 was the dominant IgE synthesis-inducing cytokine derived from highly polarized T helper (TH)₂ cells. However, anti-IL-13 mAbs also significantly inhibited IgE synthesis induced by two of three supernatants derived from allergen-specific TH₂-like cell lines generated from the skin of patients with atopic dermatitis. Furthermore, anti-IL-13 mAbs almost completely inhibited IgE synthesis induced by supernatants from TH₁ cells or CD8⁺ T cell clones. Taken together, these data indicate that IL-13, in addition to IL-4, contributes to IgE synthesis induced by all T helper cell subsets, including allergen-specific TH₂ cells. Moreover, IL-13 appears to be the major IgE synthesis-inducing cytokine derived from TH₁ cells or CD8⁺ T cells. (*J Allergy Clin Immunol* 1997;100:792-801.)

Key words: B lymphocytes, allergy, cytokines, isotype switching, T helper cells

The induction of IgE synthesis by B cells requires T cell help, which is mediated via soluble and membrane-bound molecules expressed by activated CD4⁺ T cells. The most important membrane-bound costimulatory molecule appears to be the ligand for CD40 (CD40L), which is expressed by activated CD4⁺ T cells and induces B cell proliferation and differentiation in the presence of cytokines.^{1,2} The importance of CD40L in

Abbreviations used

CD40L:	Ligand for CD40
FCS:	Fetal calf serum
IFN- γ :	Interferon- γ
IGF-1:	Insulin-like growth factor 1
mAbs:	Monoclonal antibodies
MNC:	Mononuclear cell
PBMC:	Peripheral blood mononuclear cell
T _{H0} , T _{H1} , T _{H2} :	T helper cell subsets

the induction of Ig isotype switching and Ig synthesis was demonstrated in patients with hyper-IgM syndrome, who have no or minimal levels of IgG, IgA, and IgE in their sera due to mutations in their genes encoding CD40L.³⁻⁶ The 26 kD membrane form of tumor necrosis factor- α , expressed on CD4⁺ T cells after activation, was also shown to be involved in the productive T-B cell interactions that are required for induction of IgE synthesis.⁷⁻⁸ However, IgE synthesis induced by T cells derived from patients deficient in CD40L could not be blocked by neutralizing anti-tumor necrosis factor- α monoclonal antibodies (mAbs),⁹ suggesting that additional surface-bound molecules are involved. One such a pair of molecules may be CD19 (which is constitutively expressed on B cells) and its putative ligand on T cells, because mice with CD19 deficiency have impaired T cell-dependent B cell responses.¹⁰ In addition, CD22 and CD58 expressed on B cells have also been demonstrated to mediate interactions with T cells,^{11,12} further demonstrating the complexity and redundancy involved in the regulation of productive T-B cell interactions.

Both IL-4 and IL-13 induce IgE switching and IgE synthesis by human B cells activated by CD4⁺ T cells, Epstein-Barr virus transformation, or CD40 stimulation.¹³⁻¹⁹ IL-4 and IL-13 are functionally and structurally related and they are most likely the results of gene duplication, because the genes encoding IL-4 and IL-13 are located only 12 kb from each other on chromosome 5q31.²⁰⁻²⁴ IL-13 did not have any additive or synergistic effects on IL-4-induced IgE synthesis when both cytokines were added at saturating concentrations, suggesting similarities in the signaling pathways of these two cytokines.¹⁸ Thereafter, a mutant form of IL-4 (hIL-

From the Human Immunology Department, DNAX Research Institute of Molecular and Cellular Biology, Inc., Palo Alto.

Supported by Schering-Plough Corporation and by the Academy of Finland (J.P.).

Received for publication March 7, 1997; revised June 17, 1997; accepted for publication July 2, 1997.

Reprint requests: Jan E. de Vries, PhD, Human Immunology Department, DNAX Research Institute of Molecular and Cellular Biology, Inc., 901 California Ave., Palo Alto, CA 94304-1104.

Copyright © 1997 by Mosby-Year Book, Inc.

0091-6749/97 \$5.00 + 0 1/1/84508

4.Y124D) and mAbs specific for the IL-4R α -chain were shown to block the biological functions of both IL-4 and IL-13, indicating that the IL-4R α -chain is a component of both IL-4R and IL-13R complexes.²⁴⁻²⁷ Recently, a specific IL-13 binding protein was cloned and sequenced, which, together with the IL-4R α -chain, is required for IL-13 signaling.^{28,29} Despite these similarities, not all functions of IL-4 are shared with IL-13. The major differences are that IL-13, in contrast to IL-4, does not direct type 2 T helper cell (T_{H2}) differentiation or induce proliferation by human T cells.³⁰ Moreover, IL-4, but not IL-13, induces CD23 expression and germline ϵ transcription in purified human precursor B cells, suggesting differential expression of IL-4R and IL-13R complexes during B cell ontogeny and, hence, different roles for IL-4 and IL-13 in the regulation of B cell maturation.³¹

The relative contributions of IL-4 and IL-13 to the regulation of IgE synthesis have remained poorly characterized. These studies have been hampered by the lack of suitable animal models, because IL-13 has not been shown to act on murine B cells. However, early studies indicated that IgE synthesis induced by T cells from patients with atopy is partially independent of IL-4.³² More recently, IL-13 was shown to be a primary IgE synthesis-inducing cytokine secreted by naive $CD4^+$, $CD45RO^+$ T cells.^{33,34} In addition, IL-13, but not IL-4, was shown to be required for spontaneous IgE and IgG4 synthesis by PBMC derived from patients with nephrotic syndrome.³⁵ The aim of this study was to characterize the relative contribution of IL-4 and IL-13 to T cell-dependent IgE synthesis by human B cells. We show that both anti-IL-4 and anti-IL-13 mAbs significantly inhibit IgE synthesis induced by supernatants derived from activated human $CD4^+$ T cell clones or lines. Neutralizing anti-IL-13 mAbs also inhibited IgE synthesis induced by supernatants derived from type 1 T helper cells (T_{H1}) and $CD8^+$ T cell clones, and more importantly, IL-13 contributed to IgE synthesis induced by T_{H2} -like T cell lines generated from the skin of patients with atopy. These results suggest that IL-13, in addition to IL-4, is involved in the elevated IgE synthesis observed in such individuals.

METHODS

Reagents

Purified human recombinant IL-4 was provided by Schering-Plough Research Institute (Kenilworth, N.J.). Recombinant IL-13 was provided by Dr. Satish Menon (DNAX). Unconjugated, phycoerythrin-conjugated, and fluorescein isothiocyanate-conjugated mAbs specific for CD3, CD4, CD8, CD14, CD16, CD20, CD56, and control antibodies with irrelevant specificities were obtained from Becton-Dickinson (San Jose, Calif.). The purified anti-CD40 mAb 89 (IgG1) was provided by Dr. J. Banchereau (Schering-Plough France, Dardilly, France). Neutralizing mAbs specific for IL-4 (25D2) and IL-13 (5A2) were provided by Dr. J. Abrams (DNAX). Neutralizing mAb specific for interferon- γ (IFN- γ) was purchased from R&D Systems (Minneapolis, Minn.).

Cell preparations

Blood samples and spleens were obtained from healthy volunteers or from patients undergoing splenectomy due to trauma, respectively. Spleen samples were obtained from four different donors. Mononuclear cells (MNCs) were isolated by centrifugation over Histopaque-1077 (Sigma, St. Louis, Mo.).

B cells were purified by negative selection by depleting T cells, monocytes, and natural killer cells. Briefly, splenic MNCs were stained with mAbs specific for CD3, CD4, CD8, CD14, CD16, and CD56. After the cells were washed twice, they were incubated with magnetic beads coated with anti-mouse IgG mAbs (Dynabeads M-450, Dynal, Oslo) for 30 minutes at 4°C. Positive cells were removed by a magnetic field according to the manufacturer's instructions, and the remaining cells were washed twice and resuspended in Yssel's medium¹⁸ supplemented with 10% fetal calf serum (FCS). The purity of the remaining cells was analyzed by flow cytometry, and always >97% of these cells were $CD20^+$ (data not shown).

T cell clones and lines

Thirteen $CD4^+$ T cell clones, three $CD4^+$ T cell lines derived from the skin of patients with atopy, and two $CD8^+$ T cell clones were used in this study. Four of the $CD4^+$ T cell clones were derived from cord blood.

Three T cell lines were generated from skin biopsy specimens under conditions that do not require the addition of antigen-presenting cells.³⁶ The biopsy samples were taken from patients with atopic dermatitis 24 hours after a transdermal challenge with house dust mite allergen *Dermatophagoides pteronyssinus* group 1 (Der p 1).³⁷ The cells were expanded in Yssel's medium supplemented with 1% human AB serum, IL-2 (10 U/ml), and IL-4 (100 U/ml).

Four $CD4^+$ T cell clones were generated from naive $CD4^+$, $CD45RA^+$ cord blood T cells purified as described previously.³⁰ Two of these clones were generated in the presence of IL-12, which is known to favor differentiation of T_{H1} cells.^{30,38,39} Nine $CD4^+$ T cell clones and two $CD8^+$ T cell clones were generated from the peripheral blood of healthy adult donors. All T cell clones were restimulated every other week by phytohemagglutinin and allogeneic feeder cells as described previously.³⁷

Generation of supernatants of activated T cells

A total of 23 $CD4^+$ T cell supernatants and two $CD8^+$ T cell supernatants was generated. T cell clones or lines were stimulated in 24-well plates (Lindbro, McLean, Va.) with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb. Briefly, the wells were coated with anti-CD3 mAb SPV-T3b⁴⁰ for 16 hours at 4°C, and washed twice before the addition of T cells and soluble anti-CD28 mAb L293 (a gift from Dr. L. Lanier, DNAX). The cells were cultured in Yssel's medium supplemented with 10% FCS at 2×10^6 cells/ml for 48 hours, and the supernatants were harvested, centrifuged, and stored at -80°C until used.

Induction of Ig synthesis in vitro

Unfractionated PBMC or purified B cells were cultured in quadruplicate in round-bottomed 96-well plates (Lindbro) at 10^5 cells/well at 37°C in a humidified atmosphere containing 5% CO_2 . All cultures were performed in 0.2 ml Yssel's medium supplemented with 10% FCS. Cytokines, T cell supernatants, and mAbs were added at the onset of the cultures. Ig levels in the culture supernatants were measured by specific ELISA after culture for 12 to 14 days.

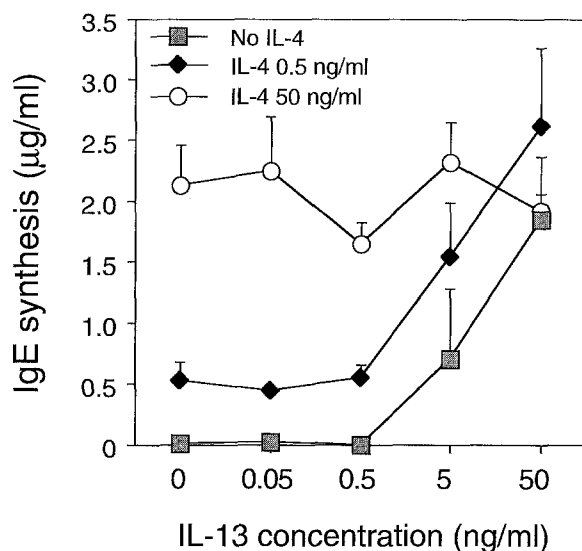


FIG. 1. IL-13 additively enhances IgE synthesis induced by sub-optimal concentrations of IL-4. Human PBMC were cultured in the presence of IL-4 (0, 0.5, or 50 ng/ml) and increasing concentrations of IL-13 were added to cultures. IgE levels in culture supernatants were measured by ELISA after culture for 12 days. Data represent mean $\pm$ SEM IgE levels of quadruplicate cultures in a representative of four experiments.

Measurement of cytokine and Ig production

IL-4 and IL-13 levels were determined by ELISA as described by Chretien et al.⁴¹ and de Waal Malefyt et al.,⁴² respectively. The sensitivity of both ELISA protocols was 50 pg/ml.

IgM, total IgG, IgG4, IgA, and IgE secretions were determined by ELISA as described previously.¹⁸ The sensitivities of IgM, total IgG, and IgA ELISAs were 0.5 ng/ml, and the sensitivities of IgG4 and IgE ELISAs were 0.2 ng/ml.

RESULTS

IL-13 has additive effects on IgE synthesis induced by suboptimal concentrations of IL-4

The effects of IL-13 on human IgE synthesis induced by increasing concentrations of IL-4 were investigated. IL-13 had no additive or synergistic effects on IL-4-induced IgE synthesis by human PBMC when IL-4 was added at saturating concentrations of 50 ng/ml (Fig. 1), which is consistent with previous results.¹⁸ However, IL-13 significantly enhanced IL-4-induced IgE synthesis in a dose-dependent manner when IL-4 was present at a suboptimal concentration of 0.5 ng/ml (Fig. 1). These results suggest that both IL-4 and IL-13 can contribute to T cell-dependent human IgE synthesis.

Neutralizing anti-IL-4 and anti-IL-13 mAbs inhibit IgE synthesis induced by CD4⁺ T cell supernatants and anti-CD40 mAbs

To investigate directly the relative contribution of IL-4 and IL-13 to polyclonal human IgE synthesis, we studied the effects of neutralizing anti-IL-4 and anti-IL-13 mAbs on IgE synthesis induced in response to anti-CD40 mAbs and supernatants of activated CD4⁺ T

cell clones. Only supernatants inducing >10 ng/ml of IgE (17 of 23) were chosen for these studies, and a total of 25 experiments was performed with the use of purified splenic B cells from four different donors. Neutralizing anti-IL-4 and anti-IL-13 mAbs inhibited CD4⁺ T cell supernatant-induced IgE synthesis by an average 61% and 42%, respectively (Fig. 2, A). In addition to IgE switching, IL-4 and IL-13 have also been shown to induce IgG4 synthesis by human B cells.^{18, 43, 44} As shown for IgE production, anti-IL-4 mAbs inhibited IgG4 synthesis induced by T cell supernatants by 52%, whereas anti-IL-13 mAbs only slightly reduced the levels of IgG4 produced (Fig. 2, A). Neither anti-IL-4 mAbs nor anti-IL-13 mAbs had any significant effect on IgM or IgA synthesis under these culture conditions (Fig. 2, B). In addition, no effect on total IgG synthesis was observed (Fig. 2, B), which is likely because IgG4 represents only a small proportion of total IgG, and reduced IgG4 production may also be compensated for by increased synthesis of other IgG subclasses. The neutralizing anti-IL-4 and anti-IL-13 mAbs we used completely blocked IgE synthesis induced by recombinant forms of the corresponding cytokines, and nonspecific inhibitory effects on IgE synthesis were not observed (Fig. 2, C).

Correlations between the levels of IL-4, IL-13, and IFN- γ production and the capacities of the T cell supernatants to induce IgE synthesis

We next studied the correlations between the concentrations of IL-4 and IL-13 in the T cell supernatants and levels of IgE synthesis induced by these supernatants in the presence of anti-CD40 mAbs. In addition, the levels of IFN- γ production were measured, because IFN- γ has direct inhibitory effects on IL-4- or IL-13-induced germ-line ϵ transcription and IgE synthesis by purified B cells.^{31, 45} (Cocks et al., submitted for publication). As expected, the level of IgE synthesis induced by T cell supernatants positively correlated with the concentrations of both IL-4 and IL-13. However, the correlation between IL-4 concentrations and the levels of IgE produced was stronger ($r = 0.423$) than that between IL-13 concentrations and IgE synthesis ($r = 0.261$) (Fig. 3, A and B), whereas a strong inverse correlation was observed between the levels of IgE produced and the concentrations of IFN- γ in the supernatants ($r = 0.567$). Therefore, supernatants with relatively low IL-4 and IL-13 levels were able to induce strong IgE responses provided that the levels of IFN- γ also were low (data not shown).

Because IL-13 is also produced by T_{H1} cells secreting high levels of IFN- γ ,⁴² we determined the correlations between the levels of IL-4, IL-13, and IFN- γ in the supernatants. As shown in Fig. 3, D, there was an inverse correlation ($r = 0.312$) between the levels of IL-4 and IFN- γ in the supernatants, whereas no correlation ($r = 0.056$) between the concentrations of IL-13 and IFN- γ was observed (Fig. 3, E). These findings are consistent with the results indicating that IL-13 can be produced by

all T helper cell subsets.⁴² However, a strong positive correlation existed between IL-4 and IL-13 production ($r = 0.646$) (Fig. 3, *F*), suggesting that IL-13 is primarily produced by T_{H2} cells, which is in line with a previous study indicating that most IL-4-producing cells also produce IL-13.³⁴ On the other hand, five of 23 supernatants contained >20 ng/ml of IL-13, but no or low levels (<2 ng/ml) of IL-4 (Fig. 3, *F*), further suggesting that IL-4 and IL-13 are partially produced by different T cell subsets and that the production of IL-4 and IL-13 is regulated by different mechanisms. Accordingly, a proportion of T cell supernatants with low levels of IL-4 induced relatively high levels of IgE synthesis (Fig. 3, *A*).

Contribution of IL-13 to IgE synthesis induced by T_{H2} -like T cell lines derived from the skin of patients with atopy

Because allergen-specific T cells generally belong to the T_{H2} subset, we studied the relative contribution of IL-4 and IL-13 to the induction of IgE synthesis by T cell lines generated from skin biopsy samples taken from patients with atopic dermatitis after a challenge with the house dust mite allergen Der p 1. Supernatants from T cell lines derived from three different patients contained 6.6 ± 3.6 (mean $\pm$ SD) ng/ml of IL-4 and 34.2 ± 9.0 ng/ml of IL-13 after activation with immobilized anti-CD3 and anti-CD28 mAbs. The production levels of IFN- γ were always <0.1 ng/ml, indicating that these cell lines were indeed highly polarized toward the T_{H2} phenotype (Fig. 4). Anti-IL-4 mAbs strongly inhibited IgE synthesis induced by all three supernatants. Neutralizing anti-IL-13 mAbs also had significant inhibitory effects on IgE synthesis induced by two of the three supernatants tested (Fig. 4, *A* and *B*). In addition, even when anti-IL-13 mAbs alone had no inhibitory effect, they could completely block the residual IgE synthesis induced by the T cell supernatant in the presence of anti-IL-4 mAbs (Fig. 4, *C*). These data suggest that IL-4 and IL-13 are the most relevant T cell-derived soluble molecules that can induce IgE synthesis by human B cells, and that IL-13, in addition to IL-4, contributes to IgE synthesis induced by allergen-specific T_{H2} cells from patients with atopy.

IL-13 is a major IgE synthesis-inducing cytokine derived from T_{H1} or $CD8^+$ T cell clones

Because IL-13, in contrast to IL-4, is also produced by T_{H1} cells,⁴² we studied the capacity of T_{H1} -like T cell clones to induce polyclonal IgE synthesis. Supernatants were derived from two adult T_{H1} -like clones and two T cell clones that had been generated from naive $CD4^+$, $CD45RA^+$ cord blood T cells in the presence of IL-12, a cytokine known to favor T_{H1} differentiation.^{30, 38, 39} Cytokine levels in the supernatants indicated that one clone derived from cord blood was a T_{H0} clone producing equal, but low, levels of IL-4 and IFN- γ (Fig. 5, *A*), whereas the other cord blood-derived and the two adult peripheral blood-derived $CD4^+$ T cell clones belonged to the T_{H1} subset with high IFN- γ /IL-4 production ratios

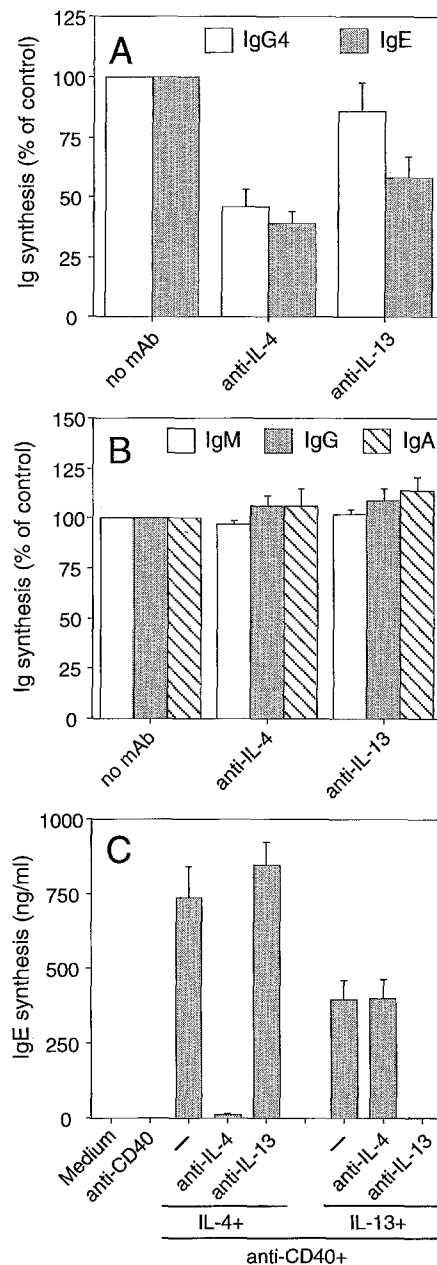


FIG. 2. Effects of neutralizing mAbs specific for IL-4 and IL-13 on IgE and IgG4 (**A**) or IgM, total IgG, and IgA (**B**) synthesis induced by supernatants of activated $CD4^+$ T cell clones, and effects of these mAbs on IgE synthesis induced by recombinant IL-4 or IL-13 (**C**). Supernatants (25% of final volume) of $CD4^+$ T cells activated by immobilized anti-CD3 and anti-CD28 mAbs (**A** and **B**), or recombinant IL-4 (100 ng/ml) or IL-13 (100 ng/ml) (**C**) were added to cultures of purified B cells stimulated with anti-CD40 mAbs (10 μ g/ml) in the presence or absence of neutralizing anti-IL-4 or anti-IL-13 mAbs (10 μ g/ml each) as indicated in the figure. Ig synthesis was studied after culture for 12 to 14 days by specific ELISAs. In **A** and **B**, 17 supernatants were studied with the use of splenic B cells from four different donors in a total of 25 experiments, and levels of Ig produced in the presence of neutralizing mAbs are expressed as percentages (mean $\pm$ SEM) of those produced in the absence of the mAbs. Mean levels of IgE, IgG4, IgM, total IgG, and IgA detected in these experiments were 230, 569, 1852, 2152, and 3566 ng/ml, respectively. In **C**, data represent mean ($\pm$ SEM) IgE levels of quadruplicate cultures in a representative of four experiments.

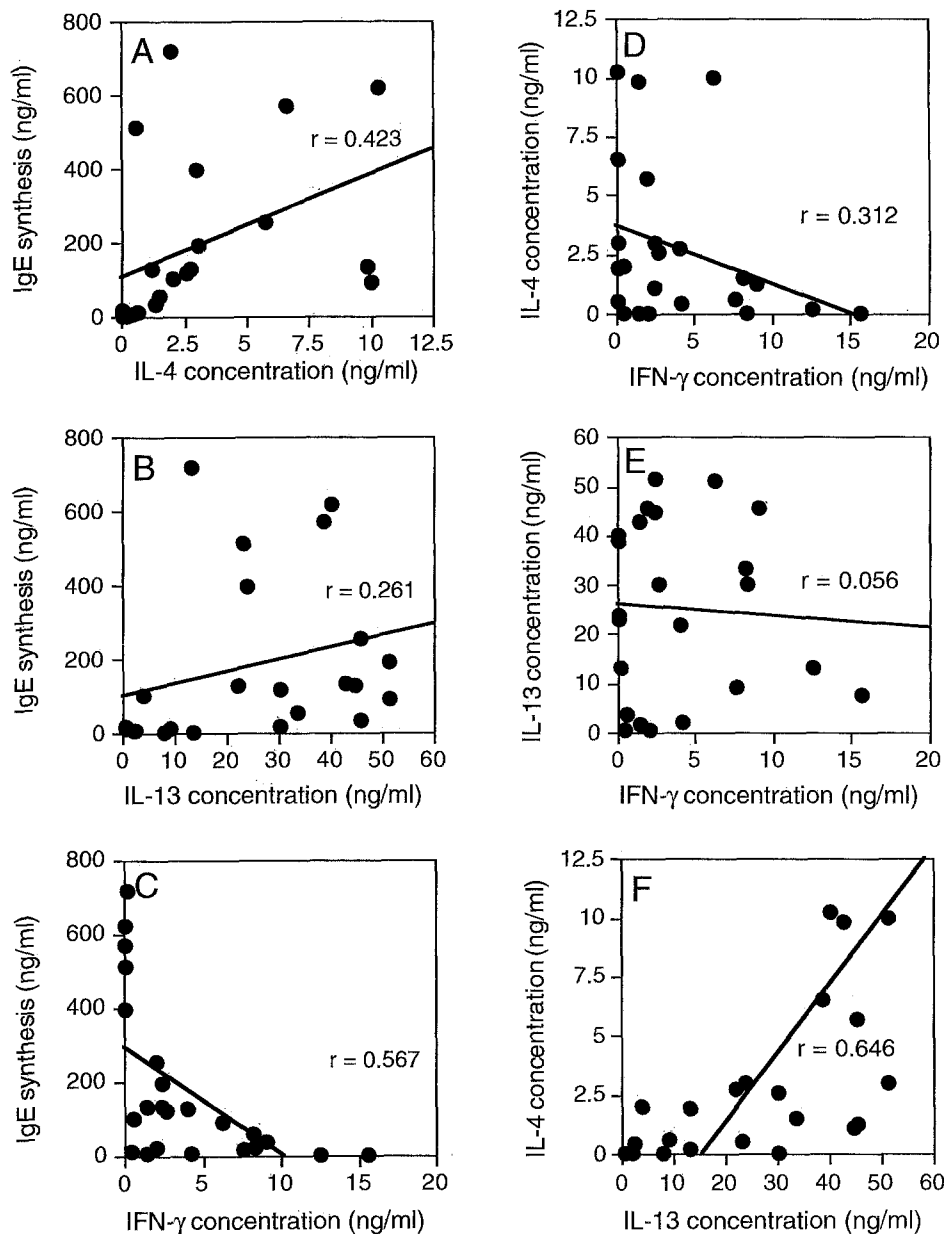


FIG. 3. Correlation of levels of IL-4 (**A**), IL-13 (**B**), and IFN- γ (**C**) in CD4 $^{+}$ T cell supernatants with the amount of IgE induced by the supernatants, and production levels of IL-4, IL-13, and IFN- γ by activated CD4 $^{+}$ T cells (**D**, **E** and **F**, respectively). Cytokine levels were studied by specific ELISAs in 23 supernatants derived from 15 different CD4 $^{+}$ T cell clones activated with immobilized anti-CD3 and anti-CD28 mAbs and the capacities of the supernatants to induce IgE were studied. Purified B cells were cultured in the presence of anti-CD40 mAbs (10 μ g/ml) and T cell supernatants (25% of final volume), and levels of IgE produced were studied after culture for 12 to 14 days.

(Fig. 5, *B*, *C*, and *D*). All four clones produced considerable levels of IL-13 (Fig. 5). Supernatants derived from these T_{H0} and T_{H1} clones were able to induce IgE synthesis by purified B cells cultured in the presence of anti-CD40 mAbs, but the levels of IgE synthesis were significantly lower than those induced by T_{H2} cells (Fig. 4). Anti-IL-13 mAbs strongly inhibited IgE production induced by all four supernatants, whereas anti-IL-4 mAbs had no or minimal inhibitory effects, except in Fig.

5, *B*. These results are consistent with the data on the levels of cytokines present in the supernatants, and indicate that IL-13 produced by T_{H0} and T_{H1} cells is a major contributor to the IgE synthesis induced by these cells.

A proportion of CD8 $^{+}$ T cells isolated from patients with human immunodeficiency virus infection who had high serum IgE levels express CD40L and produce T_{H2}-type cytokines. Supernatants derived from these

CD8⁺ T cells induced IgE synthesis by normal B cells activated with anti-CD40 mAbs.^{46, 47} As shown in Fig. 6, supernatants derived from activated CD8⁺ T cell clones obtained from healthy individuals also induced low levels of IgE synthesis by purified B cells activated with anti-CD40 mAbs. IL-4 production by these CD8⁺ T cell clones after activation with immobilized anti-CD3 and anti-CD28 mAbs was very low (<0.05 ng/ml), whereas relatively high levels of IL-13 were observed (mean 14.1 ng/ml, *n* = 2). In addition, these CD8⁺ T cells produced high levels of IFN- γ (mean 17.3 ng/ml). Accordingly, the levels of IgE produced in response to these supernatants were significantly increased when neutralizing anti-IFN- γ mAbs were included in the cultures (Fig. 6). Consistent with the results on cytokine levels in the CD8⁺ T cell supernatants, the addition of neutralizing anti-IL-4 mAbs had no significant effect, whereas anti-IL-13 mAbs virtually completely blocked IgE synthesis induced by anti-CD40 mAbs and these supernatants (Fig. 6). These data suggest that IL-13 plays a major role in the IgE synthesis induced by CD8⁺ T cell clones.

DISCUSSION

It is well documented that both IL-4 and IL-13 induce IgE synthesis by human B cells *in vitro*.¹³⁻¹⁹ However, it has remained unclear whether both cytokines also contribute to IgE synthesis under physiological situations. Initially, it was reported that mice with IL-4 deficiency are completely unable to produce IgE *in vivo*,⁴⁸ suggesting that IL-4 is the only cytokine capable of inducing IgE synthesis. However, more recent studies have indicated that low levels of IgE are produced in mice deficient in IL-4 after infections with *Plasmodium chabaudi* or *Leishmania major*, indicating that an IL-4-independent mechanism of IgE switching is operational.^{49, 50} However, whether this IgE synthesis is induced by IL-13 is not known, because IL-13 has not been reported to induce IgE switching in murine B cells. Mice deficient for Stat 6 also are unable to produce IgE *in vivo*.⁵¹ However, Stat 6 is not only involved in IL-4 signaling, but it also appears to be an essential component of the IL-13-induced signaling pathway.⁵² Consequently, no conclusions on the relative contribution of IL-4 and IL-13 to the regulation of human IgE synthesis can be drawn on the basis of the data obtained in these mouse models.

In this study, we have shown that IL-13 contributes to IgE synthesis induced by T_{H0}, T_{H1}, or T_{H2}-type CD4⁺ T cell clones, CD8⁺ T cells, and CD4⁺ T cells derived from the skin of patients with atopic dermatitis. Because recombinant IL-13 enhances IL-4-induced IgE synthesis only when IL-4 is present at suboptimal concentrations, these observations also suggest that the level of IL-4 production after T cell activation by immobilized anti-CD3 and anti-CD28 mAbs is insufficient to saturate all IL-4 receptors. However, because T cells express high-affinity IL-4R complexes, but no IL-13R complexes, it cannot be ruled out that the lower levels of IL-4 observed in the cultures were due to IL-4 consumption during T cell activation. In addition, whether the con-

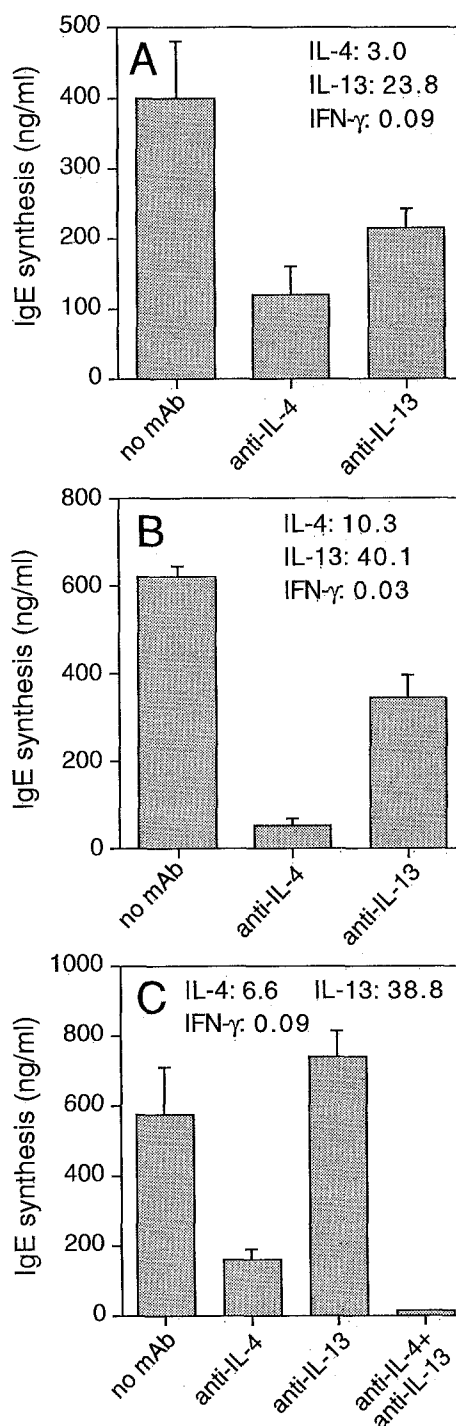


FIG. 4. Effects of neutralizing anti-IL-4 and anti-IL-13 mAbs on IgE synthesis induced in response to supernatants of CD4⁺ T cell lines derived from skin of patients with atopic dermatitis. Skin-derived T cell lines were generated after allergen challenge as described in Methods section and activated with immobilized anti-CD3 and anti-CD28 mAbs for 48 hours before harvesting the supernatants. Purified B cells (10^5 /well) were cultured in the presence of T cell supernatant (25% of final volume), anti-CD40 mAbs (10 μ g/ml), and anti-IL-4 and/or anti-IL-13 mAbs (10 μ g/ml). IgE levels in culture supernatants were measured after culture for 12 days. IL-4, IL-13, and IFN- γ levels (ng/ml) detected in each supernatant are indicated in the figure.

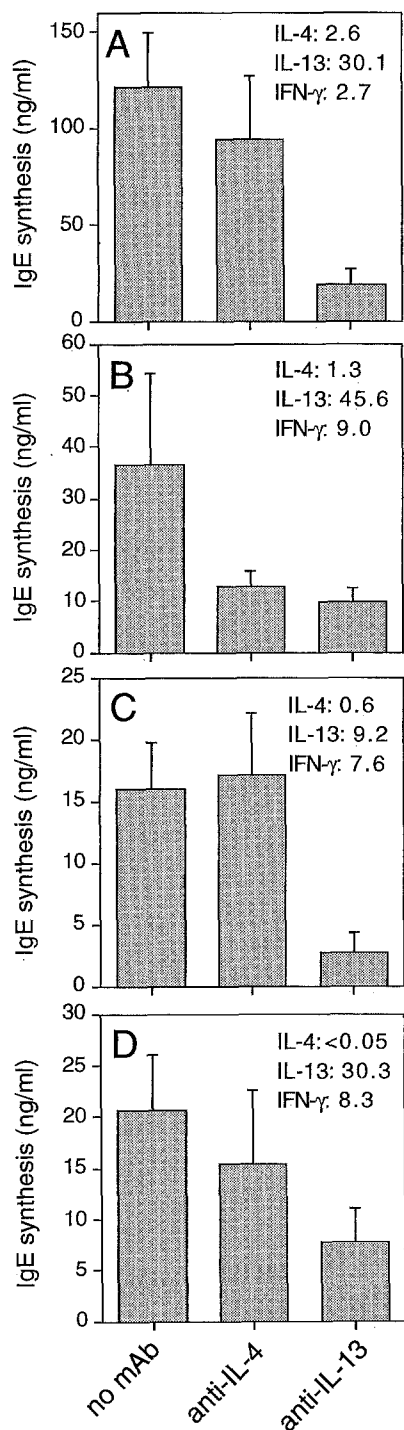


FIG. 5. Effects of neutralizing anti-IL-4 and anti-IL-13 mAbs on IgE synthesis induced in response to supernatants of T_H1 -like T cell clones. Supernatants (25% of final volume) derived from T cell clones generated from $CD4^+$ naive cord blood T cells in the presence of IL-12 (**A** and **B**) or adult T_H1 clones (**C** and **D**) were cultured in the presence of purified B cells (10^5 /well), anti-CD40 mAbs, and anti-IL-4 and/or anti-IL-13 mAbs ($10 \mu\text{g/ml}$). T cell clones were activated with immobilized mAbs anti-CD3 and anti-CD28 for 48 hours before harvesting the supernatants. IgE levels in the culture supernatants were measured after culture for 12 days. IL-4, IL-13, and IFN- γ levels (ng/ml) detected in each supernatant are indicated in the figure.

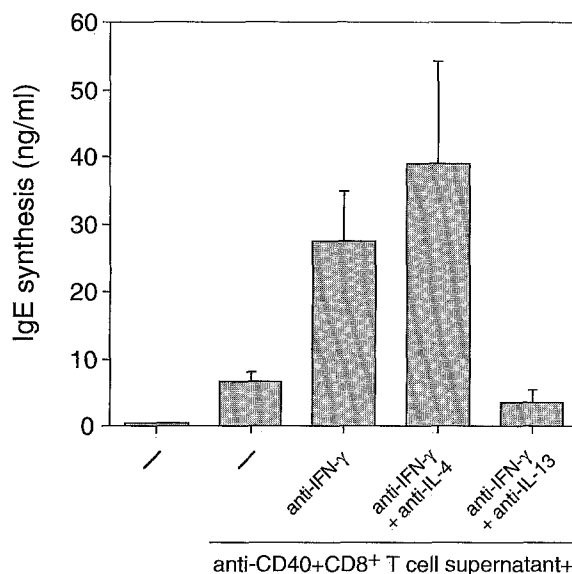


FIG. 6. Effects of neutralizing anti-IL-4 and anti-IL-13 mAbs on IgE synthesis induced by supernatants of $CD8^+$ T cell clones. $CD8^+$ T cell clones were activated with mAbs anti-CD3 and anti-CD28 for 48 hours before harvesting the supernatants. Purified B cells (10^5 /well) were cultured in the presence of T cell supernatants (25% of final volume), anti-CD40 mAbs, anti-IFN- γ , anti-IL-4, and/or anti-IL-13 mAbs ($10 \mu\text{g/ml}$ each). IgE levels in the culture supernatants were measured after culture for 12 days.

centrations of IL-4 and IL-13 required for optimal induction of IgE synthesis are similar in vitro and in vivo remains to be shown. Nevertheless, the observation that IL-4 and IL-13 have additive effects at suboptimal concentrations is consistent with studies indicating that there are two types of IL-4R complexes, one of which also acts as a high-affinity IL-13R complex. The specific high-affinity IL-4R complex comprises the common γ -chain and the IL-4R α -chain, whereas a receptor complex consisting of the IL-13R α -chain and the IL-4R α -chain acts as a receptor for both IL-4 and IL-13.^{24, 26-29} The relative contribution of the two receptor complexes in IL-4 signaling is presently unknown, but it appears that IL-13 can bind only to the receptor complexes containing the IL-13R α -chain. IL-4-induced signaling through the IL-13R complex was demonstrated in patients with X-linked severe combined immunodeficiency disease, who have mutations in their gene encoding the common γ -chain. Although these patients have defective IL-4R complexes, both IL-4 and IL-13 induced high levels of IgE synthesis through signaling via the IL-13R complex on B cells.⁵³ Our data suggest that IL-4 produced after engagement of the T-cell receptor and CD28 does not saturate all IL-4R and IL-13R complexes, and that optimal IgE responses are only obtained when IL-13, in addition to IL-4, is present.

IL-4 and IL-13 appear to be the only T cell-derived molecules capable of inducing IgE synthesis by human B cells. Activated $CD4^+$ T cells have been previously shown to induce IgE synthesis even in the presence of anti-IL-4 mAbs.³² In addition, T cell clones that did not

produce IL-4 could induce germline ϵ -transcription in human B cells.⁵⁴ In this study, anti-IL-13 mAbs could completely block the residual IgE synthesis observed in cultures treated with neutralizing anti-IL-4 mAbs, even in situations in which anti-IL-13 mAbs alone did not significantly inhibit T cell supernatant-induced IgE synthesis. However, growth hormone and insulin-like growth factor 1 (IGF-1) have also been shown to induce IgE synthesis by surface IgE-B cells cultured in the presence of anti-CD40 mAbs.⁵⁵ Because alveolar macrophages secrete IGF-1 and the levels of both growth hormone and IGF-1 are elevated in the bronchoalveolar lavage of patients with asthma,⁵⁵ it remains possible that these molecules also play a role in IL-4- and IL-13-induced IgE synthesis *in vivo*.

IL-13 was produced at high levels by T cells derived from the skin of patients with atopy, and neutralizing anti-IL-13 mAbs inhibited IgE synthesis induced by supernatants of skin-derived T cells, suggesting that IL-13 plays an important role in the regulation of atopic and allergic responses. This conclusion is supported by a study indicating that IL-13 expression, like that of IL-4, is upregulated in the lungs of patients with asthma after allergen challenge.⁵⁶ However, IL-4 and IL-13 appear to be partially produced by different T cell subsets. In our study, 5 of 24 CD4⁺ T cell supernatants contained >20 ng/ml of IL-13 but no or <2 ng/ml of IL-4, and several supernatants with high levels of IL-13 had high levels of IFN- γ . These findings are consistent with a previous study indicating that IL-13 is also produced by T_{H1} cells.⁴² Moreover, in contrast to IL-4, IL-13 was produced by naive CD45RA⁺ T cells.^{33, 34} Despite the fact that both IL-4 and IL-13 were required for optimal IgE synthesis, the levels of IgE produced correlated more significantly with IL-4 levels than with IL-13 levels in the supernatants, which is in line with the observation that IL-13 is less potent than IL-4 in inducing IgE synthesis by human B cells.^{18, 19} However, because IFN- γ produced by T_{H1} cell clones directly inhibits germline ϵ transcription and IgE synthesis^{31, 45} (Cocks et al, submitted for publication), IL-13-induced IgE synthesis was probably downregulated by IFN- γ present at high levels in a proportion of the supernatants from T_{H0}/T_{H1} cells. Nevertheless, despite the largely overlapping effects of IL-4 and IL-13 on B cell function *in vitro*, IL-13 produced by T cell subsets not producing IL-4 may play a significant role in the regulation of allergic inflammation and IgE synthesis *in vivo*. More specifically, IL-13 is likely to play a major role in the induction of IgE synthesis during the initiation of allergic responses, because IL-13, in contrast to IL-4, is also produced by naive CD45RA⁺ T cells and because the less IL-4 present, the more significant the relative contribution of IL-13.

Human CD8⁺ T cell clones infected with human immunodeficiency virus express CD40L and induce IgE synthesis by purified B cells,^{46, 47} and a subset of murine CD8⁺ T cell clones provided help for B cell growth and differentiation.⁵⁷ Supernatants from CD8⁺ T cell clones

derived from healthy individuals could also induce IgE synthesis by human B cells. However, the cytokine production profile of these T cell clones more closely resembled that of T_{H1} cells, because high levels of IFN- γ and low levels of IL-4 were produced. Supernatants from these CD8⁺ T cell clones induced low levels of polyclonal IgE synthesis by purified B cells, but the level of IgE synthesis could be significantly enhanced by neutralizing anti-IFN- γ mAbs. These supernatants contained relatively high levels of IL-13, and neutralizing anti-IL-13 mAbs completely blocked the capacity of the supernatants to induce IgE synthesis. CD8⁺ T cells have also been shown to have inhibitory effects on IgE synthesis,⁵⁸ and therefore, the contribution of CD8⁺ T cells to the induction and maintenance of IgE synthesis *in vivo* requires further study. Nevertheless, the present results suggest that IL-13 is a major CD8⁺ T cell-derived cytokine that can induce IgE synthesis by human B cells.

Taken together, both IL-4 and IL-13 contribute to IgE production induced by activated CD4⁺ or CD8⁺ T cell clones. These data emphasize the potential of molecules that block the functions of both IL-4 and IL-13 as a means to prevent IgE synthesis in individuals with allergy. More specifically, IL-4R and IL-13R antagonists, such as the mutant IL-4 (hIL-4.Y124D),²⁴⁻²⁶ may be superior to specific IL-4 antagonists, such as humanized anti-IL-4 mAbs or soluble IL-4R α -chain, in the treatment of patients with atopy.

We thank Drs. Gregorio Aversa and José M. Carballido for valuable discussions and Patricia Latchas for her expert assistance. We are grateful to Dr. Kristian Thestrup-Pedersen for providing patient samples and to Jo Ann Katheiser for careful secretarial help.

REFERENCES

1. Armitage RJ, Fanslow WC, Strockbine L, Sato TA, Clifford KN, Macduff BM, et al. Molecular and biological characterization of a murine ligand for CD40. *Nature* 1992;357:80-2.
2. Hollenbaugh D, Grosmaire LS, Kullas CD, Chalupny NJ, Braesch-Andersen S, Noelle RJ, et al. The human T cell antigen gp39, a member of the TNF gene family, is a ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell co-stimulatory activity. *EMBO J* 1992;11:4313-21.
3. Fuleihan RN, Ramesh N, Loh R, Jabara H, Rosen RS, Chatila T, et al. Defective expression of the CD40 ligand in X chromosome-linked immunoglobulin deficiency with normal or elevated IgM. *Proc Natl Acad Sci USA* 1993;90:2170-3.
4. Di Santo JP, Bonnefoy JY, Gauchat JF, Fischer A, De Saint Basile G. CD40 ligand mutations in X-linked immunodeficiency with hyper-IgM. *Nature* 1993;361:541-3.
5. Allen RC, Armitage RJ, Conley ME, Rosenblatt H, Jenkins NA, Copeland NG, et al. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science* 1993;259:990-3.
6. Aruffo A, Farrington M, Hollenbaugh D, Li X, Milatovich A, Nonoyama S, et al. The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell* 1993;72:291-300.
7. Aversa G, Punnonen J, de Vries JE. The 26 kD transmembrane form of tumor necrosis factor alpha on activated CD4⁺ T cell clones provides a costimulatory signal for human B cell activation. *J Exp Med* 1993;177:1575-85.
8. Macchia D, Almerigogna F, Parronchi P, Ravina A, Maggi E,

- Romagnani S. Membrane tumour necrosis factor- α is involved in the polyclonal B-cell activation induced by HIV-infected human T cells. *Nature* 1993;363:464-6.
9. Life P, Gauchat JF, Schnuriger V, Estoppey S, Mazzei G, Durandy A, et al. T cell clones from an X-linked hyper-immunoglobulin (IgM) patient induce IgE synthesis in vitro despite expression of nonfunctional CD40 ligand. *J Exp Med* 1994;180:1775-84.
10. Rickert RC, Rajewsky K, Roes J. Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. *Nature* 1995;376:352-5.
11. Stamenkovic I, Sgroi D, Aruffo A, Sy MS, Anderson T. The B lymphocyte adhesion molecule CD22 interacts with leukocyte common antigen CD45RO on T cells and alpha 2-6 sialyltransferase, CD75, on B cells. *Cell* 1991;66:1133-44.
12. Diaz-Sanchez D, Chegini S, Zhang K, Saxon A. CD58 (LFA-3) stimulation provides a signal for human isotype switching and IgE production distinct from CD40. *J Immunol* 1994;153:10-20.
13. Pene J, Roussel F, Briere F, Chretien I, Bonnefoy JY, Spits H, et al. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons gamma and alpha and prostaglandin E₂. *Proc Natl Acad Sci USA* 1988;85:6880-4.
14. Thyphronitis G, Tsokos GC, June CH, Levine AD, Finkelman FD. IgE secretion by Epstein-Barr virus-infected purified human B lymphocytes is stimulated by interleukin 4 and suppressed by interferon gamma. *Proc Natl Acad Sci USA* 1989;86:5580-4.
15. Vercelli D, Jabara HH, Arai K, Geha RS. Induction of human IgE synthesis requires interleukin 4 and T/B cell interactions involving the T cell receptor/CD3 complex and MHC class II antigens. *J Exp Med* 1989;169:1295-307.
16. Claassen JL, Levine AD, Buckley RH. Recombinant human IL-4 induces IgE and IgG synthesis by normal and atopic donor mononuclear cells. Similar dose response, time course, requirement for T cells, and effect of pokeweed mitogen. *J Immunol* 1990;144:2123-30.
17. Zhang K, Clark EA, Saxon A. CD40 stimulation provides an IFN-gamma-independent and IL-4-dependent differentiation signal directly to human B cells for IgE production. *J Immunol* 1991;146:1836-42.
18. Punnonen J, Aversa G, Cocks BG, McKenzie ANJ, Menon S, Zurawski G, et al. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc Natl Acad Sci USA* 1993;90:3730-4.
19. Punnonen J, de Vries JE. IL-13 induces proliferation, Ig isotype switching, and Ig synthesis by immature human fetal B cells. *J Immunol* 1994;152:1094-102.
20. McKenzie ANJ, Li ANX, Largaespada DA, Sato A, Kaneda A, Zurawski SM, et al. Structural comparison and chromosomal localization of the human and mouse IL-13 genes. *J Immunol* 1993;150:5436-44.
21. Smirnov DV, Smirnova MG, Korobko G, Frolova EL. Tandem arrangement of human genes for interleukin-4 and interleukin-13: resemblance in their organization. *Gene* 1995;155:277-81.
22. Minty A, Chalon P, Derocq JM, Dumont X, Guillemot JC, Kaghad M, et al. Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. *Nature* 1993;362:248-50.
23. McKenzie ANJ, Culpepper JA, de Waal Malefyt R, Briere F, Punnonen J, Aversa G, et al. Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. *Proc Natl Acad Sci USA* 1993;90:3735-9.
24. Zurawski SM, Vega Jr F, Huyghe B, Zurawski G. Receptors for interleukin-13 and interleukin-4 are complex and share a novel component that functions in signal transduction. *EMBO J* 1993;12:2663-70.
25. Kruse N, Tony HP, Sebald W. Conversion of human interleukin-4 into a high affinity antagonist by a single amino acid replacement. *EMBO J* 1992;11:3237-44.
26. Aversa G, Punnonen J, Cocks BG, de Waal Malefyt R, Vega Jr FJ, Zurawski SM, et al. An interleukin 4 (IL-4) mutant protein inhibits both IL-4 or IL-13-induced human immunoglobulin G4 (IgG4) and IgE synthesis and B cell proliferation: support for a common component shared by IL-4 and IL-13 receptors. *J Exp Med* 1993;178:2213-8.
27. Zurawski SM, Chomarat P, Djossou O, Bidaud C, McKenzie ANJ, Miossec P, et al. The primary binding subunit of the human interleukin-4 receptor is also a component of the interleukin-13 receptor. *J Biol Chem* 1995;270:13869-78.
28. Hilton DJ, Zhang JG, Metcalf D, Alexander WS, Nicola NA, Willson TA. Cloning and characterization of a binding subunit of the interleukin 13 receptor that is also a component of the interleukin 4 receptor. *Proc Natl Acad Sci USA* 1996;93:497-501.
29. Caput D, Laurent P, Kaghad M, Lelias JM, Lefort S, Vita N, et al. Cloning and characterization of a specific interleukin (IL)-13 binding protein structurally related to the IL-5 receptor alpha-chain. *J Biol Chem* 1996;271:16921-6.
30. Sornasse T, Larenas PV, Davis KA, de Vries JE, Yssel H. Differentiation and stability of T helper 1 and 2 cells derived from naive human neonatal CD4⁺ T cells, analyzed at the single-cell level. *J Exp Med* 1996;184:473-83.
31. Punnonen J, Cocks BG, de Vries JE. IL-4 induces germline IgE heavy chain gene transcription in human fetal pre-B cells: evidence for differential expression of functional IL-4 and IL-13 receptors during B cell ontogeny. *J Immunol* 1995;155:4248-54.
32. Zhang X, Polla B, Hauser C, Zubler RH. T cells from atopic individuals produce IgE-inducing activity incompletely blocked by anti-interleukin-4 antibody. *Eur J Immunol* 1992;22:829-33.
33. Brinkmann V, Kristofic C. TCR-stimulated naive human CD4⁺ 45RO-T cells develop into effector cells that secrete IL-13, IL-5, and IFN-gamma, but no IL-4, and help efficient IgE production by B cells. *J Immunol* 1995;154:3078-87.
34. Jung T, Wijdenes J, Neumann C, de Vries JE, Yssel H. Interleukin-13 is produced by activated human CD45RA⁺ and CD45RO⁺ T cells: modulation by interleukin-4 and interleukin-12. *Eur J Immunol* 1996;26:571-7.
35. Kimata H, Fujimoto M, Furusho K. Involvement of interleukin (IL)-3, but not IL-4, in spontaneous IgE and IgG4 production in nephrotic syndrome. *Eur J Immunol* 1995;25:1497-501.
36. Kaltoft K, Hansen BH, Pedersen CB, Pedersen S, Thestrup-Pedersen K. Common clonal chromosome aberrations in cytokine-dependent continuous human T-lymphocyte cell lines. *Cancer Genet Cytogenet* 1995;85:68-71.
37. Yssel H, Johnson KE, Schneider PV, Wideman J, Terr A, Kastelein R, et al. T cell activation-inducing epitopes of the house dust mite allergen Der p I. Proliferation and lymphokine production patterns by Der p I-specific CD4⁺ T cell clones. *J Immunol* 1992;148:738-45.
38. Manetti R, Parronchi P, Giudizi MG, Piccinini MP, Maggi E, Trinchieri G, et al. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J Exp Med* 1993;177:1199-1204.
39. Marshall JD, Secrist H, De Kruffy RH, Wolf SF, Umetsu DT. IL-12 inhibits the production of IL-4 and IL-10 in allergen-specific human CD4⁺ T lymphocytes. *J Immunol* 1995;155:111-7.
40. Spits H, Keizer G, Borst J, Terhorst C, Hekman A, de Vries JE. Characterization of monoclonal antibodies against cell surface molecules associated with cytotoxic activity of natural and activated killer cells and cloned CTL lines. *Hybridoma* 1983;2:423.
41. Chretien I, Van Kimmenade A, Pearce M, Banchereau J, Abrams JS. Development of polyclonal and monoclonal antibodies for immunoassay and neutralization of human interleukin-4. *J Immunol Methods* 1989;117:6781.
42. de Waal Malefyt R, Abram JS, Zurawski SM, Lecron JC, Mohan-Peterson S, Sanjanwala B, et al. Differential regulation of IL-13 and IL-4 production by human CD8⁺ and CD4⁺ Th0, Th1 and Th2 T cell clones and EBV-transformed B cells. *Int Immunol* 1995;7:1405-16.
43. Gascan H, Gauchat JF, Roncarolo MG, Yssel H, Spits S, de Vries JE. Human B cell clones can be induced to proliferate and to switch to IgE and IgG4 synthesis by interleukin 4 and a signal provided by activated CD4⁺ T cell clones. *J Exp Med* 1991;173:747-50.
44. Lundgren M, Persson U, Larsson P, Magnusson C, Smith CI, Hammarstrom L, et al. Interleukin 4 induces synthesis of IgE and IgG4 in human B cells. *Eur J Immunol* 1989;19:1311-5.
45. Xu L, Rothman P. IFN-gamma represses epsilon germline transcription and subsequently down-regulates switch recombination to epsilon. *Int Immunol* 1994;6:515-21.

46. Paganelli R, Scala E, Ansotegui IJ, Ausiello CM, Halapi E, Fanale-Belasio E, et al. CD8⁺ T lymphocytes provide helper activity for IgE synthesis in human immunodeficiency virus-infected patients with hyper-IgE. *J Exp Med* 1995;181:423-8.
47. Maggi E, Giudizi MG, Biagiotti R, Annunziato F, Manetti R, Piccinni MP, et al. Th2-like CD8⁺ T cells showing B cell helper function and reduced cytolytic activity in human immunodeficiency virus type 1 infection. *J Exp Med* 1994;180:489-95.
48. Kuhn R, Rajewsky K, Muller W. Generation and analysis of interleukin-4 deficient mice. *Science* 1991;254:707-10.
49. vonder Weld T, Kopf M, Kohler G, Langhorne J. The immune response to *Plasmodium chabaudi* malaria in interleukin-4-deficient mice. *Eur J Immunol* 1994;24:2285-93.
50. Noben-Trauth N, Kropf P, Muller I. Susceptibility to *Leishmania major* infection in interleukin-4-deficient mice. *Science* 1996;271:987-90.
51. Shimoda K, Kvan Deursen J, Sangster MY, Sarawar SR, Carson RT, Tripp RA, et al. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat 6 gene. *Nature* 1996;380:630-3.
52. Lin JX, Migone TS, Tsang M, Friedmann M, Weatherbee JA, Zhou L, et al. The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-5. *Immunity* 1995;2:331-9.
53. Matthew DJ, Clark PA, Herbert J, Morgan G, Armitage RJ, Kinnon C, et al. Function of the interleukin-2 (IL-2) receptor gamma-chain in biologic responses of X-linked severe combined immunodeficient B cells to IL-2, IL-4, IL-3, and IL-15. *Blood* 1995;85:38-42.
54. Gauchat JF, Lebman DA, Coffman RL, Gascan H, de Vries JE. Structure and expression of germline epsilon transcripts in human B cells induced by interleukin 4 to switch to IgE production. *J Exp Med* 1990;172:463-73.
55. Kimata H, Fujimoto M. Growth hormone and insulin-like growth factor I induce immunoglobulin (Ig)E and IgG4 production by human B cells. *J Exp Med* 1994;180:727-32.
56. Huang SK, Xiao HQ, Kleine-Tebbe J, Paciotti G, Marsh DG, Lichtenstein LM, et al. IL-13 expression at the sites of allergen challenge in patients with asthma. *J Immunol* 1995;155:2688-94.
57. Cronin DC, Stack R, Fitch FW. IL-4-producing CD8⁺ T cell clones can provide B cell help. *J Immunol* 1995;154:3118-27.
58. Kemeny DM, Noble A, Holmes BJ, Diaz-Sanchez D. Immune regulation: a new role for the CD8⁺ T cell. *Immunol Today* 1994;15:107-10.

Your comprehensive reference to valuable research information...

Now you can have easy access to research information with the five-year cumulative author and subject index to Volumes 87-96 of *The Journal of Allergy and Clinical Immunology*.

The Cumulative Index is your comprehensive reference guide, including a list of every article in the Journal from 1991 through 1995. All articles are indexed both by subject and by author.

The Cumulative Index features:

- Offset and bold Subject Headings that make searching for subject entries easier
- Subject Entries containing complete article title, author(s), year of publication, volume, and page
- Author Entries listing author(s), article title, author-to-author referral, year of publication, volume, and page

Order *The Journal of Allergy and Clinical Immunology* Cumulative Index today!

M Mosby

YES! Please send me the Five-Year Cumulative Index to *The Journal of Allergy and Clinical Immunology* (1991-1995). I've indicated my method of payment below. All prices include shipping.

☐ \$55.00 U.S. ☐ \$63.66 Canada* ☐ \$59.50 International

*Includes Canadian GST.

Payment must accompany your order.

☐ Check enclosed (U.S. funds, payable to *The Journal of Allergy and Clinical Immunology*)

☐ MasterCard ☐ VISA ☐ Discover

Credit Card # _____ Exp. Date _____

Signature _____

Name _____

Institution _____

Address _____

City _____ State _____

ZIP _____ Country _____

To order, just call

1-800-453-4351

Outside the U.S.: 314-453-4351

Fax: 314-432-1158

Or return coupon to:

Mosby-Year Book, Inc.
Journal Subscription Services
11830 Westline Industrial Dr.
St. Louis, MO 63146 U.S.A.

J01976Z