

The role of IL-12 in the induction of late-phase cellular infiltration in a murine model of allergic conjunctivitis

M. Teresa Magone, MD,^a Scott M. Whitcup, MD,^a Atsuki Fukushima, MD, PhD,^{a,b}
Chi-Chao Chan, MD,^a Phyllis B. Silver, BA,^a and Luiz Vicente Rizzo, MD, PhD^{a,c}
Bethesda, Md, Kochi, Japan, and São Paulo, Brazil

Background: The applied murine model of allergic conjunctivitis mimics human disease, and an immediate hypersensitivity reaction (IHR) and a late-phase cellular reaction typically develop in sensitized mice after topical challenge with the allergen.

Objective: We investigated the role of IL-4, IFN- γ , and IL-12 in the early and late phases of ocular allergy with use of cytokine knockout (KO) mice and neutralizing antibodies.

Methods: Ragweed-sensitized wild-type or IL-4KO, IL-12KO, IFN- γ KO, anti-IL-12 mAb-treated, recombinant murine IL-12-treated, and anti-IFN- γ mAb-treated mice were challenged with the allergen 10 days after the immunization. IHR, cellular infiltration, lymphoproliferative response, and cytokine production from draining lymph nodes were recorded and compared among groups.

Results: We show that IL-12KO mice and anti-IL-12 antibody-treated wild-type animals failed to have a cellular infiltration into the conjunctiva. Treatment with recombinant murine IL-12 also reduced the number of infiltrating PMNs but increased the percentage of mononuclear cells in the conjunctiva compared with controls. IFN- γ KO mice had a significantly stronger IHR and prolonged infiltration into the conjunctiva after challenge with ragweed than controls.

Conclusion: Our data suggest that the presence of IL-12, although better known as a T_H1-inducing cytokine, is important for the development and the regulation of the late-phase pathologic features in ocular allergy. Furthermore, IFN- γ is a limiting factor in the late phase of allergy and thus may be important in preventing chronic allergic disease. (*J Allergy Clin Immunol* 2000;105:299-308.)

Key words: IL-12, IFN- γ , allergic conjunctivitis, cytokine-deficient mice, IL-4

Seasonal allergic conjunctivitis (SAC) is one of the most common forms of allergic reactions in humans; it affects roughly 3 million people in the United States alone.¹⁻³ The conjunctiva is exposed to a number of putative allergens from the environment and SAC develops in susceptible individuals. The disease is characterized by an early phase associated with palpebral and conjunctival

Abbreviations used

Fc ϵ R:	High-affinity Fc ϵ receptor
IHR:	Immediate hypersensitivity reaction
IP:	Intraperitoneal
KO:	Knockout
rm:	Recombinant murine
RW:	Short ragweed
SAC:	Seasonal allergic conjunctivitis
Th:	T-helper cell
WT:	Wild type

edema and mild mucus discharge, as well as a late-phase response 6 to 24 hours after exposure to the allergen.⁴ This late phase is characterized by the accumulation of neutrophils and eosinophils in the conjunctiva.⁵⁻⁷ Although a substantial amount of knowledge has been gained regarding the early phase of allergic disease, the exact mechanisms driving cellular infiltration into the conjunctiva are still not entirely understood. It has been clear for a number of years that the acute-phase response is mediated by antibodies, mostly IgE, that occupy the high-affinity Fc receptors (Fc ϵ R1) on the surface of mast cells. Subsequently, on cross-linking by the allergen, these FcR-bound IgEs induce mast cell degranulation. Activated CD4⁺ T cells have been proposed to orchestrate the late-phase reaction in asthma models through the release of different T-helper (T_H) type-2 cytokines such as IL-4 and IL-5, which contribute to inflammation.^{8,9} IL-3 and TNF- α have also been implicated in the pathogenesis of the late-phase allergic reaction.¹⁰⁻¹² Because of the implication that T_H2-type cytokines are involved in both the acute phase through the induction of IgE and the late phase, it has been thought that T_H1-type cytokines may have an inhibitory effect in the development of allergies. Among the classic T_H1-type cytokine, IFN- γ has been shown to inhibit secondary allergic responses in mice.^{13,14} Because it is known that IL-12 is a potent inducer of IFN- γ secretion, the role of this cytokine in allergic reactions has also been studied. Recent publications have shown that IL-12 is a strong inhibitor of T_H2-type cytokine expression and that treatment with exogenous IL-12 reduced allergic disease in mice.¹⁵⁻²⁰ In addition, IL-12 also reduces IgE production in vitro.²¹ Nevertheless, we and others have shown that IL-12 synergizes with IL-4 in inducing cell proliferation and even IgE synthesis.^{22,23} These apparent contradictory

From the ^aNational Eye Institute, National Institutes of Health, Bethesda, Md, ^bKochi Medical School, Kochi, Japan, and the ^cUniversity of São Paulo, São Paulo, Brazil.

Received for publication May 26, 1999; revised Oct 19, 1999; accepted for publication Oct 19, 1999.

Reprint requests: M. Teresa Magone, MD, National Eye Institute, National Institutes of Health, Bldg 10, Rm 10N208, Bethesda, MD 20892-1858.

1/1/103827

results highlight the need to better understand the role of these and other cytokines in the development of allergies.

To investigate the role of IL-4, IFN- γ , and IL-12 in the early and late phases of ocular allergy, we used a previously described murine model of allergic conjunctivitis that parallels human disease.²⁴ We examined whether IL-12- or IFN- γ -deficient mice had enhanced allergic disease in the early or late phases of disease and whether we could observe a shift in cytokine production in lymphocytes from ragweed-sensitized IFN- γ - and IL-12-deficient mice compared with their normal littermates. We have also examined whether exogenous IL-12 treatment reduced disease in the current model. Our data suggest that the presence of IL-12, although better known as a T_H1-inducing cytokine, is important for the development and the regulation of the late-phase pathologic features in ocular allergy.

MATERIAL AND METHODS

Animals

Six- to 12-week-old IFN- γ knockout (KO) and wild-type (WT) (C57BL/6) littermate mice were obtained from Jackson Laboratories (Bar Harbor, Me). IL-4 KO and IL-12 p40 KO mice were bred in house homozygously from founders graciously provided by Dr Renate A. Morawetz (National Institute of Allergy and Infectious Diseases, National Institutes of Health) and Dr Jeanne Magran (Hoffman LaRoche).²⁵ In IL-12 p40 KO mice, IL-12 was not detected by ELISA assay nor by Western blot with use of the anti-p40 mAb. We performed ELISA as described in the Material and methods section with the C15.6 (2.5 μ g/mL) for capture and the biotinylated C17.8 antibody (5 μ g/mL) for detection. We also performed Western blot analysis of the supernatants from lymphocyte cultures with use of the C15.1 mAb. All cytokine-deficient mice had a C57BL/6 background. Mice were kept in microisolator cages under specific pathogen-free conditions and were handled in compliance with National Institutes of Health and Association for Research in Vision and Ophthalmology guidelines for animal use.

Reagents

All reagents were obtained as previously described.²⁴

Immunization protocol and induction of allergic conjunctivitis

As previously described, short ragweed (RW) and alum were mixed at a concentration of 50 μ g of RW pollen in 5 mg of alum 40 minutes before immunization.²⁴ On day 0 mice were immunized with RW and alum into one hind footpad. On day 11, conjunctivitis was induced by topical application of 2 mg of RW pollen suspended in 10 μ L of PBS, pH 7.2, into each eye. In SWR/J and BALB/C mice this immunization and challenge protocol induces a strong clinical reaction, followed by cellular infiltration into the conjunctiva. C57BL/6 mice typically have a low clinical reaction and fewer cells infiltrating the conjunctiva.

Antibodies and cytokine treatment protocols

Anti-mouse IFN- γ antibodies for in vivo experiments were purchased from Endogen. Mice were treated twice with 150 μ g of anti-IFN- γ mAb intraperitoneally (IP) on day 10 after the immunization first at 15 hours before the challenge with topical RW and then at 24 hours after challenge. Control mice were treated with isotype-matched rat antibody (Endogen, Woburn, Mass). Antimouse IL-12 antibodies were produced from the hybridoma C17.8 kindly pro-

vided by Dr Giorgio Trinchieri (Wistar Institute, Philadelphia, Pa). WT mice were given IP injections of antimurine IL-12 (1 mg/mouse/treatment) or rat IgG on day -1 before immunization and days 1 and 3 after the immunization. Control mice were treated with an equal dose of rat IgG obtained from Sigma. Recombinant mouse (rm) IL-12 (R&D System, Minneapolis, Minn) or PBS was given IP at a dosage of 100 ng daily either at immunization and daily until day 4 (early treatment) or from day 5 through day 9 (late treatment).

Clinical evaluation

Animal eyes were photographed and examined clinically for signs of immediate hypersensitivity response 20 minutes after the topical application of RW. Chemosis, conjunctival redness, and lid edema were each graded on a 0 to 4+ scale. The total score consisted of the sum of scores in each of these 4 categories. The highest score of 12 was given to eyes that had severe edema in conjunctival and lid tissue and redness from vasodilation, a score of 8 was given to eyes with moderate chemosis and lid edema, and a score of 4 was given to eyes with mild conjunctival edema and erythema.²⁴ A score of 2 was given for minimal edema and erythema. Clinical evaluation and photographs were compared by a masked observer, and a final score was given to each eye. The final results show the mean score of both eyes of each mouse.

Histologic examination

Eyes were enucleated with the attached lids and intact conjunctiva. The eyes were immediately processed as previously described.²⁴ Six conjunctival tissue sections from each individual eye were obtained and examined. The number of infiltrating neutrophils, eosinophils, and mast cells were counted in 6 sections from different parts of the conjunctiva. The best time point to examine the eyes of the animals was determined previously.²⁴ In our model a cellular infiltrate will start to develop in mice 3 hours after challenge. However, unlike in patients, the peak of the cellular response will occur around 24 hours after the topical challenge. This applies also to C57BL/6 mice. Therefore cellular infiltration in different mouse strains was compared at 24 hours and at several time points thereafter.

Lymphocyte proliferation assay

Draining popliteal lymph node cells were collected on day 11 and pooled within each group. Proliferative responses to 0.15 to 15 μ g/mL of RW extract were tested. The cells (3×10^6 /mL) were cultured in flat-bottomed microplates in a final volume of 0.2 mL of RPMI 1640 medium supplemented with HEPES buffer 25 mmol/L, 2 mmol/L L-glutamine, 5×10^{-5} mol/L 2-mercaptoethanol and HL-1 (Hycor Biomedical, Irvine, Calif). The cultures were incubated for a total of 90 hours and pulsed with tritiated thymidine (0.5 μ Ci/10 μ L/well) during the last 16 hours, harvested on a Tomtec harvester (Tomtec, Orange, Conn), and counted with a scintillation counter (Wallac, Turku, Finland). The results are presented as the mean counts per minute of tritiated thymidine incorporation from 3 separate assays.

Lymphocyte culture for cytokine analysis

Lymphocytes from draining popliteal lymph nodes were collected 10 days after immunization and cultured in RPMI 1640 medium supplemented with HEPES buffer 25 mmol/L, 2 mmol/L L-glutamine, 5×10^{-5} mol/L 2-ME, 50 μ g/mL gentamicin sulfate, and 0.5% fresh-frozen mouse serum. Lymphocytes were stimulated with medium alone, RW extract (15 μ g/mL), or concavalin A (1 μ g/mL). Supernatants were collected at 48 hours and stored at -70°C until cytokine analysis was performed. Cytokine levels in the

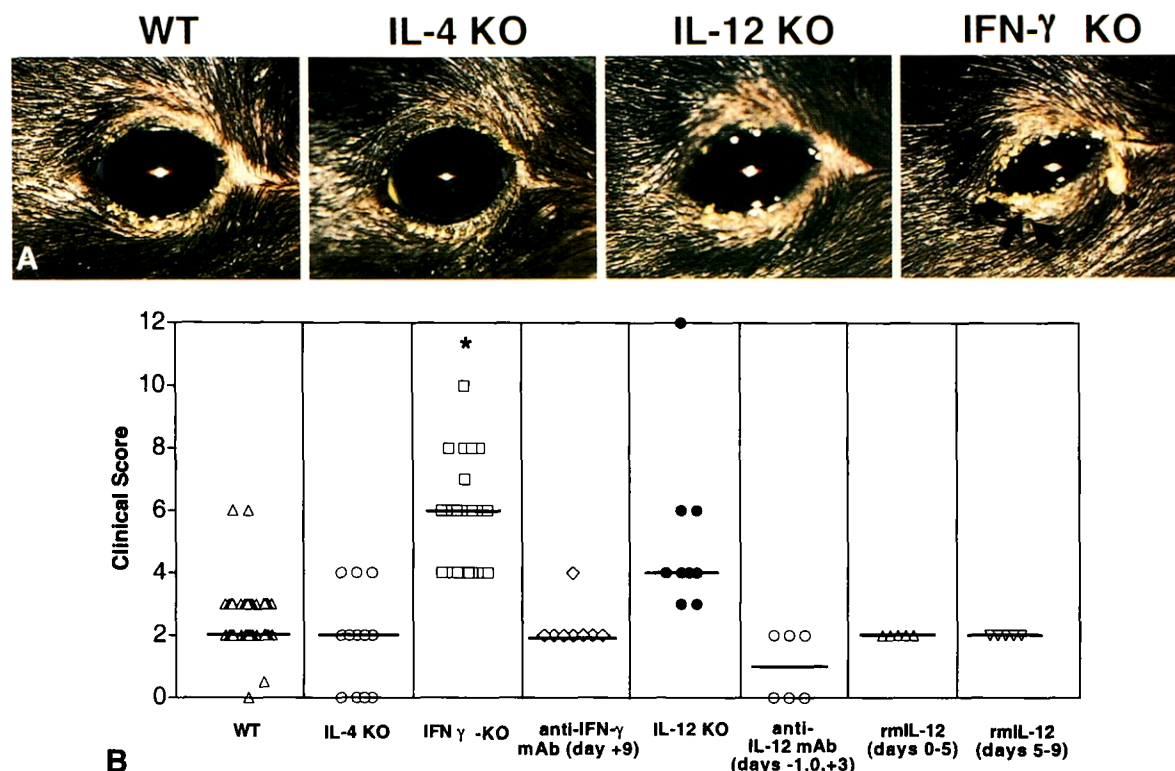


FIG 1. IHR in IL-4 KO, IL-12 KO, IFN- γ KO mice, and WT control mice. **A,** Representative mouse eyes 20 minutes after challenge with RW pollen. WT C57BL/6 mouse and IL-4 KO mouse have minimal acute-phase hypersensitivity. IL-12 KO mouse has moderate lid edema and discharge. IFN- γ KO mouse has lid edema (arrow), tearing, and discharge (arrow tip). **B,** Scatter graph of median scores of IHR. One dot represents mean of both eyes of each mouse. C57/BL6 WT mice and IL-4 KO mice were not susceptible to the acute phase of ocular allergy, whereas IFN- γ KO mice had significantly higher degrees of disease ($P < .001$). IL-12 KO mice had mildly increased disease, whereas anti-IL-12-treated mice and rmlL-12-treated mice had no disease increase. Lines, Median disease scores.

supernatants were measured with use of an ELISA as previously described before.²⁶ Briefly, IFN- γ and IL-4 were measured with antibody pairs obtained from Pharmingen (La Jolla, Calif). Biologic activity in units or concentration in picograms per milliliter was calculated from a standard curve established with the respective recombinant cytokine. IL-5 was measured with use of a minikit from Endogen (Woburn, Mass).

Western blot analysis of supernatants from IL-12 p40 KO mice

PHA-stimulated spleen cells had their supernatants collected 24 and 48 hours after stimulation. Supernatants were diluted 1:2, 1:4, 1:16, and 1:32 and were run on a 12% SDS-PAGE gel. The immunoblot sandwich was prepared as described by Coligan et al.²⁷

Detection of serum antibodies

Sera collected 11 days after immunization were analyzed by ELISA to measure the levels of total IgE, IgG1, and IgG2a. Total IgE, IgG1, and IgG2a assays were detected as previously described.^{24,28}

Statistical analysis

All results are presented as the mean $\pm$ SE. Comparisons between groups were done with use of ANOVA, and the P values are given where appropriate.

RESULTS

Challenge with RW pollen induces an immediate-type response in the eye in IFN- γ - and IL-12-deficient mice but not in IL-4-deficient mice

Ten days after immunization with RW in alum, IL-4- (n = 13), IL-12KO- (n = 10), IFN- γ -deficient mice (n = 28) and WT control mice (n = 15, n = 10, n = 22, respectively) were challenged with 2 mg of RW in 10 μ l of PBS onto each eye. In this model susceptible strains of mice immunized with RW and alum develop an immediate hypersensitivity reaction (IHR) characterized by lid edema, conjunctival redness, and chemosis as well as tearing and scratching of the eye lids.²⁴ This reaction peaks at 20 minutes after the challenge, which was the time point for evaluation and grading. WT C57Bl/6 mice are not highly susceptible to allergic conjunctivitis and had a median clinical score of 2 (0-12). IL-4 KO mice had a score of 2, and IL-12 KO mice had a median clinical score of 4. However, IFN- γ KO mice had a median score of 6, which was significantly higher than that of WT controls ($P < .001$) (Fig 1). Mice treated with anti-

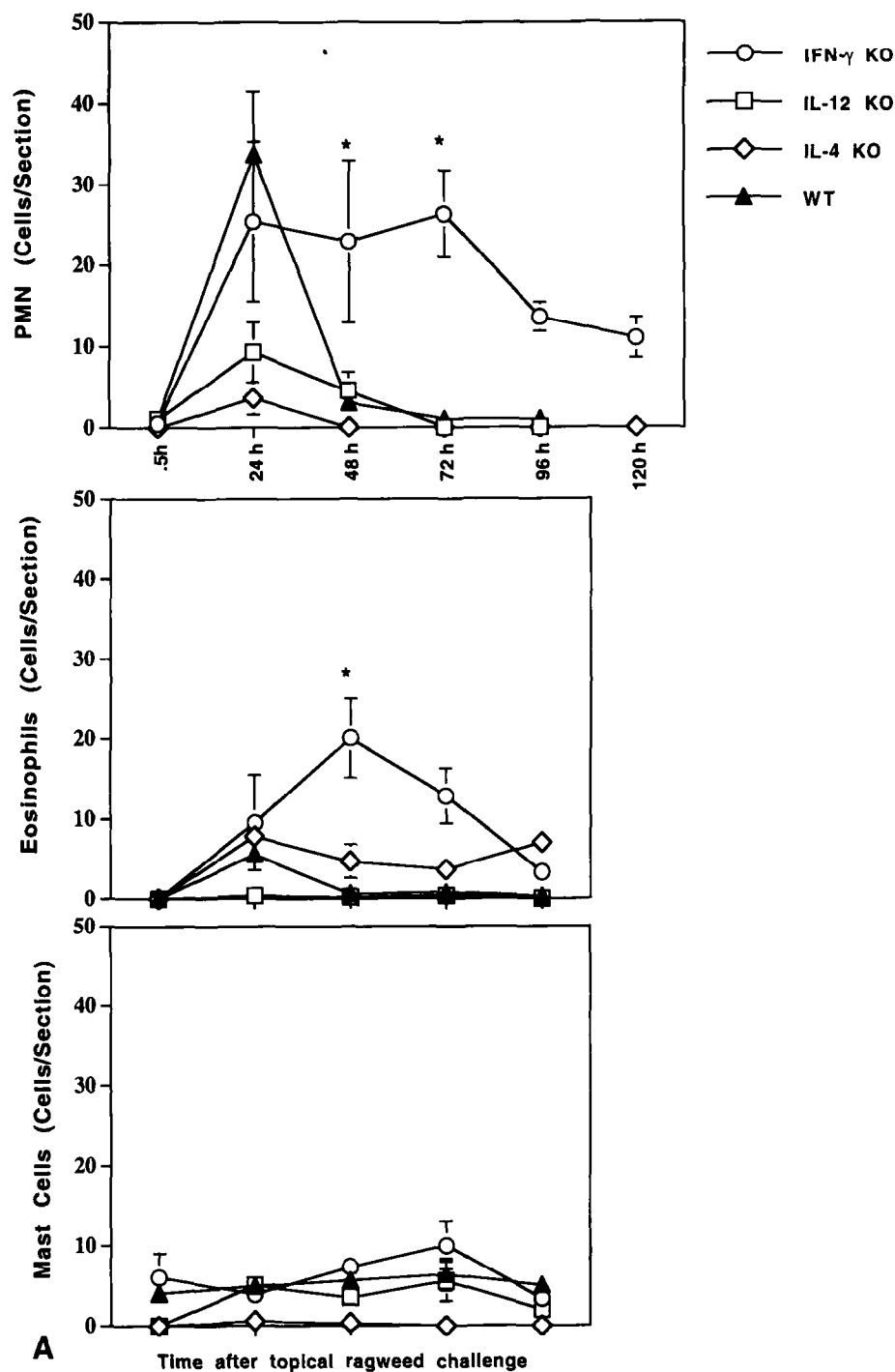


FIG 2. Cellular infiltration into conjunctiva 0.5, 24, 48, 72, 96, and 120 hours after topical challenge with RW in sensitized mice. **A**, Infiltration of polymorphonuclear cells, eosinophils, and mast cells over time in WT mice, IL-4 KO mice, IFN- γ KO mice, and IL-12 KO mice. IL-4 KO (open diamonds) and IL-12 KO mice (open squares) did not have cellular infiltration into conjunctiva, whereas IFN- γ KO mice (open circles) had prolonged PMN infiltration and more eosinophilic infiltration than WT mice did (closed triangles) ($P = .02$). **B**, Cellular infiltration 24 hours after challenge in WT mice, anti-IL-12-treated mice, IL-12 KO mice, and rIL-12-treated mice (day 0-5 or day 5-9). Cellular infiltration is significantly reduced in IL-12 KO mice ($P = .03$), anti-IL-12-treated mice ($P = .001$), and rIL-12-treated mice (day 0-5, $P = .04$) compared with WT controls. **C**, Microphotographs of conjunctiva 24 hours after topical challenge with ragweed: WT mice (**A**) show moderate infiltration of polymorphonuclear cells into conjunctiva. IL-4 KO mice (**B**) do not show cellular infiltration, whereas IFN- γ KO mice (**C**) have dense cellular infiltration with many eosinophils (arrows). IL-12 KO mice (**D**) have only minimal infiltration into conjunctiva. Treatment with anti-IFN- γ mAb (**E**) before challenge resulted in increased cellular infiltration compared with WT mice (**A**). In contrast, treatment with anti-IL-12 mAbs (**F**) reduced infiltration into conjunctiva. Also, mice treated with rIL-12 at the time of sensitization (day 0-5) (**G**) or before challenge (day 5-9) (**H**) had reduced infiltration with PMNs but a higher percentage of mononuclear cells in conjunctiva (arrows) compared with controls. (Giemsa stain, original magnification $\times 400$.)

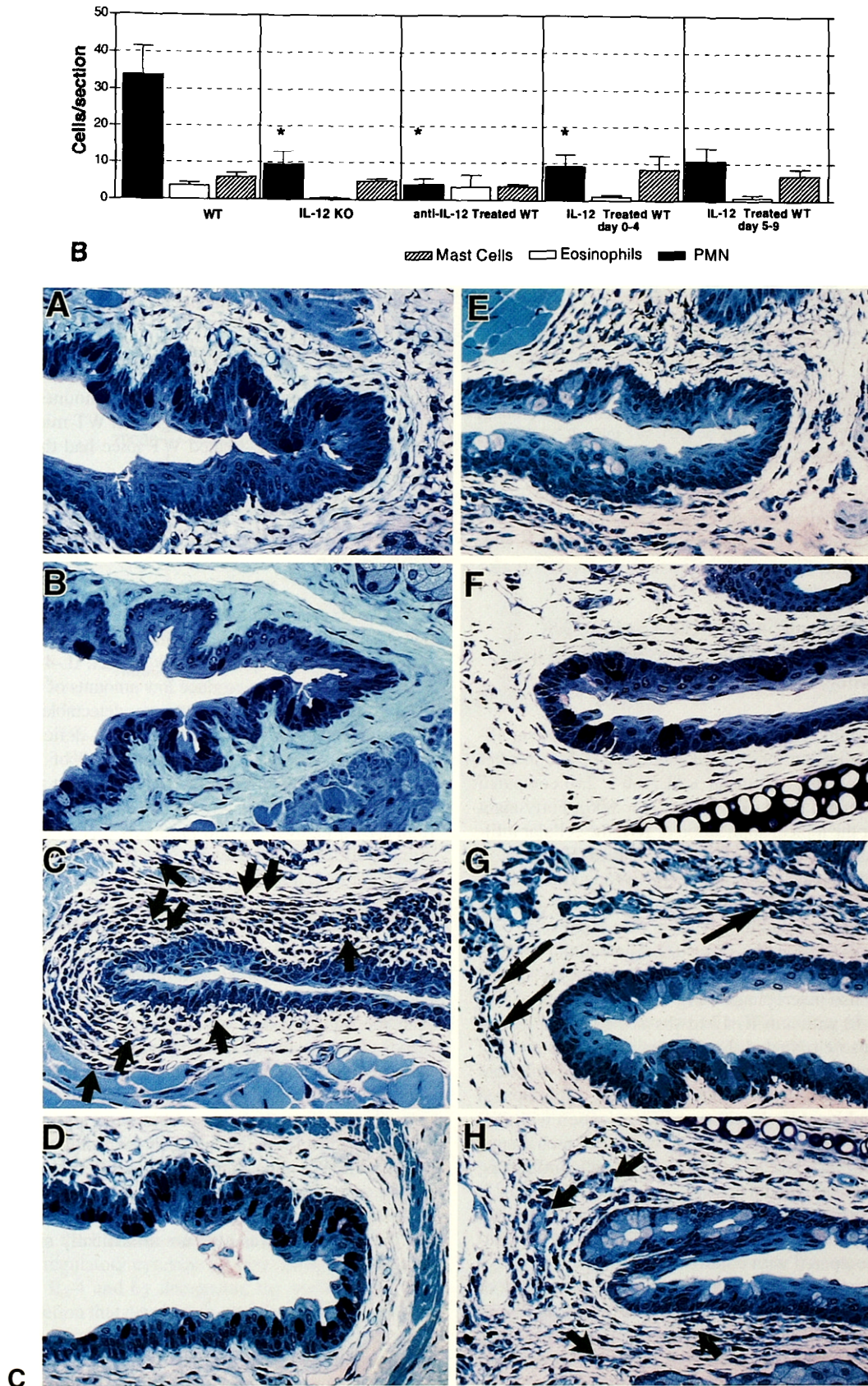


FIG 2. CONT.

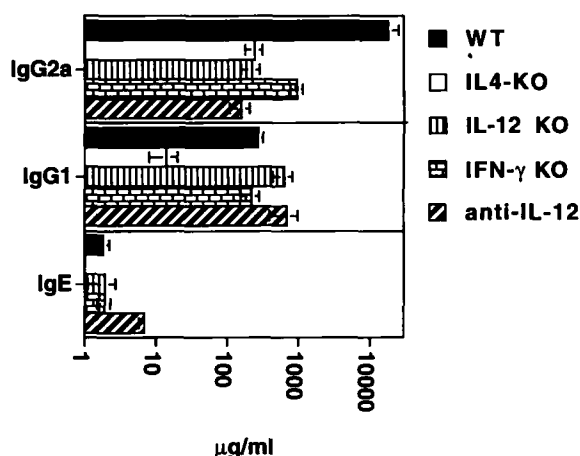


FIG 3. Total serum IgE, IgG1, and IgG2a in IL-4 KO, IL-12 KO, and IFN- γ KO mice and WT control mice: IL-4 KO mice did not produce IgE and only minimal amounts of IgG1. WT mice, IL-12 KO, and IFN- γ KO mice produced similar levels of IgE.

IFN- γ mAbs had a score of 4, anti-IL-12-treated ($n = 10$) mice had a score of 2, and rIL-12-treated mice with early as well as late treatments ($n = 6$, respectively) also had a median score of 2.

Cellular infiltration into the conjunctiva persists up to 72 hours after challenge in IFN- γ -deficient mice

As shown in Fig 2, IL-4-deficient mice had no significant cellular infiltration into the conjunctiva 24 hours after the allergen challenge (mean $\pm$ SE, 3.6 ± 2.0) compared with WT control mice (33.7 ± 7.8 , $P = .006$). Forty-eight hours after the topical application of RW, the cellular infiltration had subsided (3.0 ± 1.0) in WT mice (Fig 2, A).

IL-12-deficient mice, despite IgE production and an IHR, had significantly less cellular infiltration into the conjunctiva after challenge (9.3 ± 3.7) compared with WT controls (33.7 ± 7.8 , $P = .03$). The cellular infiltration consisted predominantly of PMNs, eosinophils, mast cells, and also macrophages (Fig 2, A). Treatment of WT mice ($n = 6$) with anti-IL-12 mAbs at the time of immunization also eliminated the cellular infiltration into the conjunctiva (4.0 ± 1.0) compared with isotype-matched controls (27.3 ± 5.1 , $P = .001$) (Fig 2, B). Mice treated with recombinant IL-12 either at immunization with RW and alum (9.2 ± 3.2 , $P = .04$) or at the time of rechallenge (10.6 ± 3.8) also had lower numbers of cells infiltrating the conjunctiva than did control mice (24.0 ± 6.8) (Fig 2, B). However, 60% (3/5) of the early treatment group had an increase in mononuclear cell infiltration in the conjunctiva compared with controls (Fig 2, C).

In contrast, IFN- γ -deficient mice had an intensity of cellular infiltration into the conjunctiva similar to that of WT mice, which was, however, longer lasting and remained the same 48 hours (26.3 ± 5.4 , $P = .06$) and 72 hours (25.4 ± 8.3 , $P = .04$) after the challenge. This infiltration persisted up to 120 hours after the single topical

administration of the allergen. In addition, IFN- γ -deficient mice had a significantly larger number of infiltrating eosinophils at 48 hours after the challenge (24.0 ± 5.0) compared with controls (1.5 ± 0.5 , $P = .02$). This eosinophilia peaked at 48 hours after the challenge and remained until 72 hours after the challenge ($P = .01$) (Fig 2, A). In an independent separate experiment we immunized C57/BL6 mice ($n = 7$) with RW and alum and treated them with anti-IFN- γ mAbs 15 hours before the topical challenge with RW pollen and then every 48 hours. Similarly, those mice also showed a delay in cellular infiltration mainly with PMNs (30.6 ± 12.7), which peaked at 72 hours and then decreased and was back to normal at 120 hours.

Total IgE levels were comparable in IFN- γ -deficient mice and IL-12-deficient mice. As expected, IL-4-deficient mice produced only minimal amounts of IgE. Serum IgG1 and IgG2a were higher in WT mice than in KO mice. Anti-IL-12-treated WT mice had the highest levels of total IgE (Fig 3).

Cytokine production in IFN- γ - and IL-12-deficient mice does not shift toward the T_H2 -type profile

WT mice produced high amounts of IL-4 (780 ± 55.7 pg/mL) and IL-5 (225 ± 49.3 pg/mL) but also IFN- γ (6147 ± 3784 pg/mL) in response to RW extract. IL-4-deficient mice were only able to produce low amounts of IL-5 (127 ± 9 pg/mL) but did not produce any detectable levels of IFN- γ . Neither the IL-12 nor the IFN- γ -deficient mice shifted their response to the production of T_H2 -type cytokines. Lymphocytes from IL-12-deficient mice produced high levels of IL-4 (403 ± 135 pg/mL) and IL-5 (336 ± 87.5 pg/mL) but still produced IFN- γ (195 ± 53.5 pg/mL). IFN- γ KO mice produced low levels of IL-4 (200.5 ± 126.2) and IL-5 (154 ± 65.6 pg/mL) (Fig 4).

IL-12 reduces lymphocyte proliferation to RW extract

We examined the lymphoproliferative response of lymphocytes from popliteal, draining lymph nodes 11 days after the immunization with RW and alum. The lowest response to RW extract was observed in WT mice treated systemically with rIL-12 at the time of immunization with RW and alum. Mice treated with rIL-12 had a statistically significantly lower proliferation compared with IL-12 KO mice ($P = .04$) and a lower proliferation compared with WT mice (however, this difference was not statistically significant; $P = .06$, ANOVA). Anti-IL-12-treated mice had the highest response to RW extract, but the difference was also not statistically significant (Fig 5).

DISCUSSION

Emerging evidence shows that allergic diseases, including ocular allergy, are not only regulated by humoral components but also by T_H2 -type CD4 $^{+}$ T

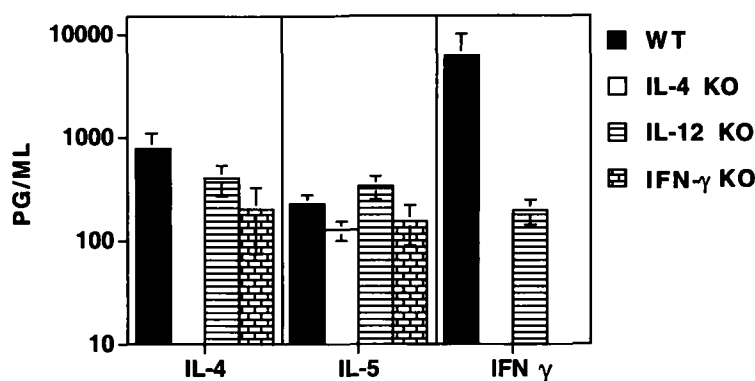


FIG 4. Bar graph of cytokine profiles in IL-4 KO, IFN-γ KO, and IL-12 KO mice. After stimulation of draining lymphocytes for 48 hours with RW extract, as described in Material and methods, cytokine profiles in IL-12 KO and IFN-γ KO mice do not shift toward T_H2 .

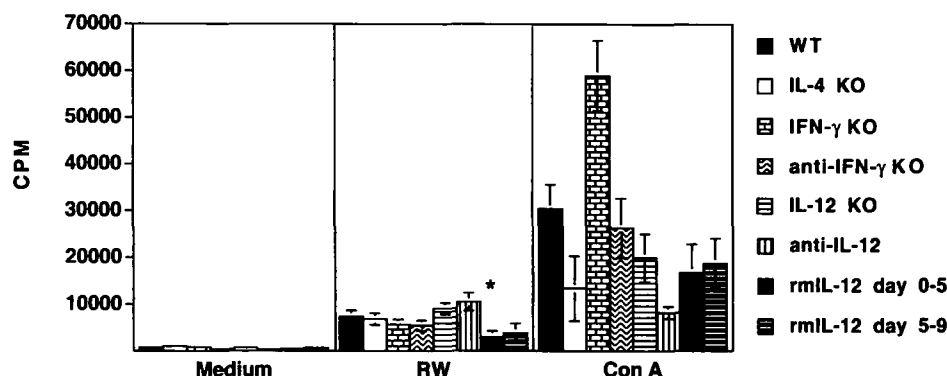


FIG 5. Lymphoproliferative response to RW extract in cultures from draining lymph nodes. Systemic rmlL-12 therapy reduced proliferation compared with IL-12 KO mice ($P = .04$). Anti-IL-12-treated mice had levels comparable to those of WT control mice.

cells.^{8,29-32} Reduction of disease by shifting responses from T_H2 into T_H1 either by treatment with T_H1 cytokines such as IFN-γ and IL-12 or with T_H1 cytokine-inducing oligonucleotide sequences has been demonstrated by several groups.^{17,18,33-35} On the other hand, Hansen et al³⁶ recently demonstrated that allergen-specific T_H1 cells do not counterbalance airway hyperreactivity in a model of asthma but may even cause severe inflammation, especially if allergen-specific T_H2 cells are also present. The major goal of the current study was to examine the role of the T_H1 cytokines IFN-γ and IL-12 in the early and late phases of allergic conjunctivitis.

IFN-γ is known to inhibit the development of allergic reactions.^{13,14,37} We have shown exacerbation of the disease in the absence of IFN-γ (IFN-γ-deficient mice or animals treated with anti-IFN-γ). Our results reinforce the data in the literature suggesting that IFN-γ may function as a regulatory cytokine by both decreasing the synthesis of IL-4 and by decreasing the eosinophilic late-phase reaction that develops in allergic reactions. We have previously shown that in the absence of IFN-γ even a typical T_H1 response such as experimental autoimmune uveitis can be modified to include an extensive eosinophilic infiltrate.³⁸ Together, these results suggest that one

of the most important functions of IFN-γ is related to controlling the migration of neutrophils and eosinophils to the site of the hypersensitivity reaction. In fact, patients with asthma were reported to produce less IFN-γ and IL-12 than controls, which could predispose them to prolonged infiltration and chronic disease.¹⁹ The precise mechanism by which IFN-γ acts on eosinophils is yet unknown, and it can either be by decreasing proliferation directly at the inflammatory site or indirectly by decreasing the synthesis of IL-5 and other eosinophilic factors or by decreasing the response of cells to IL-5 and other substances.^{39,40} These data are important because eosinophils have been repeatedly implicated in tissue damage and induction of chronic allergic disease.^{3,41,42} However, neutrophils and lymphocytes are capable of inducing airway hypersensitivity in the absence of eosinophils.⁴³

IL-12 is a cytokine mostly described to play a role in T_H1 type diseases, and exogenous treatment with IL-12 inhibits allergen-induced T_H2 -mediated airway inflammation through the induction of IFN-γ production.^{15,17,18} However, in the presence of small amounts of IL-4, IL-12 was shown to enhance T_H2 cell development.⁴⁴ In addition, the amount of IL-12, as well as the time point at which IL-12 is introduced to the tissue, can enhance or

inhibit an immune response.^{33,35} Brusselle et al³⁵ treated WT mice in a model of airway hypersensitivity with recombinant IL-12 at the time of immunization or at the time of re-exposure to the allergen. Similar to our observations, cellular infiltration was reduced in both cases. In addition, the same authors described a significant increase in macrophages after exogenous IL-12 treatment. Similarly, in our model 60% of the IL-12-treated mice had an increase in mononuclear cells in the conjunctiva despite the decrease in PMN infiltration. This observation suggests a shift in the cellular response to the cytokine. IL-12 is a key cytokine involved in multiple steps during inflammation. An excess amount of IL-12 from exogenous treatment, especially at the time of immunization, can shift the T-cell profile from T_H2 to T_H1 , whereas the lack of IL-12 can inhibit the development of the late phase in airway hypersensitivity. Although treatment with IL-12 reduces disease, our data suggest that the presence of IL-12 is important for the development of the late phase of ocular allergy. Thus the lack of this cytokine during the development of effector T cells prevents cellular infiltration into the conjunctiva. T_H2 CD4⁺ T cells can induce IL-12 production from dendritic cells, and in addition, IL-12 does not suppress a T_H2 type recall response, which supports the theory of IL-12 involvement in the pathogenesis of allergy.^{45,46} Recently, it was also shown that the development of effector T_H1 , but also T_H2 cells, seem to be dependent on the presence of IL-12.⁴⁷ In T_H1 -mediated autoimmunity it was shown that IL-12-deficient mice are not susceptible to experimental autoimmune uveitis.^{48,49} However, anti-IL-12-treated WT mice were only resistant to disease if the treatment was implemented at the time of immunization, when effector cells were being activated (Silver P, personal communication). Similarly, in our model of T_H2 -mediated disease, the presence of IL-12 was necessary for the late-phase response, which is thought to be mediated by CD4⁺ T cells.^{31,50} A recent study shows that histamine, a proinflammatory mediator released by mast cells during the acute phase of allergy, inhibits the production of IL-12.⁵¹ This inhibition was shown to be IFN- γ independent and could serve as a T_H2 -enhancing mechanism but at the same time could also prevent further effector cell development, which takes place in immune system recall responses. As mentioned earlier, Hansen et al³⁶ demonstrated that allergen-specific T_H1 cells given in the presence of allergen-specific T_H2 cells do not suppress disease but cause severe inflammation in airway hypersensitivity. It is possible that IL-12 produced by the T_H1 cells enhanced the development of T_H2 effector cells. In our study IL-12 KO mice had slightly increased IHR scores (median 4) compared with WT mice and anti-IL-12-treated mice (median 2) despite their significant decrease in cellular infiltration. This increase in the IHR in IL-12 KO mice points toward different regulatory mechanisms in the early and late phases of allergy and also to a complex regulation of histamine-related responses. IL-12 KO mice have a significantly reduced ability to produce IFN- γ , although they

can produce IL-10, a T_H2 -associated cytokine. Histamine, a mediator that is released by mast cells on degranulation, plays an essential role in IHR: it inhibits IL-12 and stimulates IL-10 production.⁵² However, although histamine enhances IFN- γ production in cell lines from healthy subjects, it fails to do so in cells from presensitized patients.⁵³ This shows that the response to mediators such as histamine, but also the production of IFN- γ , is different in presensitized allergic individuals, as previously discussed. In contrast to IL-12 KO mice, anti-IL-12-treated mice probably have the ability to produce higher levels of protective IFN- γ , which results in an unelevated IHR. Furthermore, the response to histamine is reduced when mice are treated with CTLA-4 Ig in a model of allergic rhinitis, which demonstrates that intercellular interactions are continuing and necessary after histamine release to induce a full allergic response.⁵⁴ Interestingly, IL-18, a relatively new cytokine that is thought to enhance mainly T_H1 -type disease, was also shown to enhance allergy enhancing cytokines such as IL-13.⁵⁵ This observation together with our findings suggests that the T_H1 - T_H2 paradigm is not characterized by separated pathways but by complex overlapping interactions between them. Thus the initiation of a T_H2 response is dependent on T_H1 -type cytokines such as IL-12 and, in addition, also dependent on IFN- γ for its limitation. Furthermore, IL-12 was shown to cooperate with IL-4 for proliferation, which further points toward an involvement of this assumed T_H1 cytokine in allergic diseases.²² It has been well documented that the induction of IgE responses requires the presence of IL-4.^{56,57} However, IL-4-deficient mice were shown to be able to produce IgE when infected with *Leishmania major* or *Plasmodium chabaudi*.^{58,59} Our results in the IL-4-deficient mice confirmed the previous observation that the development of allergy in certain mouse strains, such as the C57Bl/6 strain, is dependent on IL-4, although it may not be in other strains.⁶⁰ Other evidence for additional factors involved in the pathogenesis of allergy is the ability to induce an acute-phase response in sensitized IgE or mast cell-deficient mice.⁶¹⁻⁶³

In summary, the ability of the immune system to compensate, initiate, or limit responses to T_H1 - or T_H2 -mediated diseases is still under intense investigation. We have shown that IFN- γ has a protective effect on the late-phase cellular infiltration of ocular allergy. In addition, we found that IL-12, a cytokine predominantly implicated with T_H1 -type diseases, is important for the development of effector cells in the late phase of ocular allergy. These findings together with our results suggest a more complex, interactive, and important role of T_H1 -type cytokines in allergy than previously assumed.

REFERENCES

1. Trocme SD, Raizman MB, Bartley GB. Medical therapy for ocular allergy. *Mayo Clin Proc* 1992;67:557-65.
2. Ehlers WH, Donshik PC. Allergic ocular disorders: a spectrum of diseases. *Claio J* 1992;18:117-24.
3. Foster CS. The pathophysiology of ocular allergy: current thinking. *Allergy* 1995;50:6-9; discussion 34-8.

4. Reiss J, Abelson MB, George MA, Wedner HJ. Allergic conjunctivitis. In: Pepose JS, Holland GN, Wilhelmus KR, editors. Ocular infection and immunity. St Louis: Mosby; 1996:345-58.
5. Bonini S, Vecchione A, Naim DM, Allansmith MR, Balsano F. Inflammatory changes in conjunctival scrapings after allergen provocation in humans. *J Allergy Clin Immunol* 1988;82:462-9.
6. Abelson MB, Udell JJ, Weston JH. Conjunctival eosinophils in compound 48/80 rabbit model. *Arch Ophthalmol* 1983;101:631-3.
7. Anderson DF, MacLeod JD, Baddeley SM, et al. Seasonal allergic conjunctivitis is accompanied by increased mast cell numbers in the absence of leucocyte infiltration. *Clin Exp Allergy* 1997;27:1060-6.
8. Nakajima H, Iwamoto I, Tomoe S, et al. CD4+ T-lymphocytes and interleukin-5 mediate antigen-induced eosinophil infiltration into the mouse trachea. *Am Rev Respir Dis* 1992;146:374-7.
9. Gavett SH, Chen X, Finkelman F, Wills-Karp M. Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *Am J Respir Cell Mol Biol* 1994;10:587-93.
10. Kung TT, Stelts D, Zurcher JA, et al. Mast cells modulate allergic pulmonary eosinophilia in mice. *Am J Respir Cell Mol Biol* 1995;12:404-9.
11. Belser RB, Fine ED, Boehm KD. Role of tumor necrosis factor and granulocyte-macrophage colony-stimulating factor in the late allergic response in human nasal mucosa. *Otolaryngol Head Neck Surg* 1996;114:418-23.
12. Levi-Schaffer F, Temkin V, Malamud V, Feld S, Silberman Y. Mast cells enhance eosinophil survival in vitro: role of TNF-alpha and granulocyte-macrophage colony-stimulating factor. *J Immunol* 1998;160:5554-62.
13. Lack G, Bradley KL, Hamelmann E, et al. Nebulized IFN-gamma inhibits the development of secondary allergic responses in mice. *J Immunol* 1996;157:1432-9.
14. Nakajima H, Iwamoto I, Yoshida S. Aerosolized recombinant interferon gamma prevents antigen-induced eosinophil recruitment in mouse trachea. *Am Rev Respir Dis* 1993;148:1102-4.
15. Gavett SH, O'Hearn DJ, Li X, Huang S-K, Finkelman FD, Wills-Karp M. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J Exp Med* 1995;182:1527-36.
16. Hamid QA, Schotman E, Jacobson MR, Walker SM, Durham SR. Increases in IL-12 messenger RNA+ cells accompany inhibition of allergen-induced late skin responses after successful grass pollen immunotherapy. *J Allergy Clin Immunol* 1997;99:254-60.
17. Kips JC, Brusselle GJ, Joos GF, et al. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness in mice. *Am J Respir Crit Care Med* 1996;153:535-9.
18. Schwarze J, Hamelmann E, Cieslewicz G, et al. Local treatment with IL-12 is an effective inhibitor of airway hyperresponsiveness and lung eosinophilia after airway challenge in sensitized mice. *J Allergy Clin Immunol* 1998;102:86-93.
19. van der Pouw Kraan TCTM, Boeijs LCM, de Groot ER, et al. Reduced production of IL-12 and IL-12 dependent IFN-gamma release in patients with allergic asthma. *J Immunol* 1997;158:5560-5.
20. Keane-Myers A, Wysocka M, Trinchieri G, Wills-Karp M. Resistance to antigen-induced airway hyperresponsiveness requires endogenous production of IL-12. *J Immunol* 1998;161:919-26.
21. Kuniwa M, Gately M, Gubler U, Chizzonite R, Fargeas C, Delespesse G. Recombinant interleukin-12 suppresses the synthesis of immunoglobulin E by interleukin-4 stimulated human lymphocytes. *J Clin Invest* 1992;90:262-6.
22. Xu H, Rizzo LV, Caspi RR. IL-12 induces growth of the IL-4-dependent CT4S line and has a synergistic effect on IL-4-induced CT4S proliferation. *J Immunol Methods* 1995;181:245-51.
23. Wu CY, Demeure CE, Gately M, et al. In vitro maturation of human neonatal CD4 T lymphocytes. I: induction of IL-4-producing cells after long-term culture in the presence of IL-4 plus either IL-2 or IL-12. *J Immunol* 1994;152:1141-53.
24. Magone MT, Rizzo LV, Chan CC, Kozhich AT, Whitcup SM. A new murine model of allergic conjunctivitis. *Clin Immunol Immunopathol* 1998;87:75-84.
25. Magram J, Connaughton SE, Warrier RR, et al. IL-12 deficient mice are defective in IFN gamma production and type 1 cytokine responses. *Immunity* 1996;4:471-81.
26. Rizzo LV, Miller-Rivero NE, Chan CC, Wiggert B, Nussenblatt RB, Caspi RR. Interleukin-2 treatment potentiates induction of oral tolerance in a murine model of autoimmunity. *J Clin Invest* 1994;94:1668-72.
27. Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, editors. Current protocols in immunology. New York: John Wiley; 1991.
28. Rizzo LV, De Kruyff RH, Umetsu DT, Caspi RR. Regulation of the interaction between Th1 and Th2 T cell clones to provide help for antibody production in vivo. *Eur J Immunol* 1995;25:708-16.
29. Aebischer I, Stadler BM. Th1-Th2 cells in allergic responses. *Adv Immunol* 1996;61:341-403.
30. Romagnani S. The Th1/Th2 paradigm. *Immunol Today* 1997;18:263-6.
31. Galli SJ, Lantz CS. Allergy. In: Paul WE, editor. Fundamental immunology. 4th ed. Philadelphia: Lippincott-Raven; 1998. p 1123-74.
32. Tomoe S, Iwamoto I, Yoshida S, Tomioka H. The in vivo depletion of CD4+ T cells prevents antigen-induced eosinophil infiltration into mouse skin. *Arenugi* 1992;41:699-703.
33. Sur S, Lam J, Bouchard P, Sigounas A, Holbert D, Metzger J. Immunomodulatory effects of IL-12 on allergic lung inflammation dependent on timing of doses. *J Immunol* 1996;157:4173-80.
34. Broide D, Schwarze J, Tighe H, et al. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J Immunol* 1998;161:7054-62.
35. Brusselle GG, Kips JC, Peleman RA, et al. Role of IFN-gamma in the inhibition of the allergic airway inflammation caused by IL-12. *Am J Respir Cell Mol Biol* 1997;17:767-71.
36. Hansen G, Berry G, DeKruyff RH, Umetsu DT. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J Clin Invest* 1999;103:175-83.
37. Shi H, Zhong X, Long X, Chen Y. Effect of interferon-gamma on antigen-induced eosinophil infiltration into the mouse airway. *Chin Med J (Engl)* 1996;109:215-8.
38. Jones LS, Rizzo LV, Agarwal RK, et al. IFN-gamma deficient mice develop experimental autoimmune uveitis in the context of a deviant effector response. *J Immunol* 1997;158:5997-6005.
39. Rizzo LV, Secord EA, Tsiagbe VK, et al. Components essential for the generation of germinal centers. *Dev Immunol* 1998;6:325-30.
40. Rizzo LV. Differential modulatory effect of IL-5 on MHC class II expression by macrophages and B cells. *Braz J Med Biol Res* 1992;25:509-13.
41. Hingorani M, Calder V, Jolly G, Buckley RJ, Lightman SL. Eosinophil surface antigen expression and cytokine production vary in different ocular allergic diseases. *J Allergy Clin Immunol* 1998;102:821-30.
42. Makino S, Fukuda T. Eosinophils and allergy in asthma. *Allergy Proc* 1995;16:13-21.
43. Walsh GM, Hartnell A, Wardlaw AJ, Kurihara K, Sanderson CJ, Kay AB. IL-5 enhances the in vitro adhesion of human eosinophils, but not neutrophils, in a leukocyte integrin (CD11b/18)-dependent manner. *Immunology* 1990;71:258-65.
44. Ruede E, Schmitt E, Germann T. IL-12 and its receptor. In: Delves PJ, Roitt IM, editors. Encyclopedia of immunology. Vol 3. San Diego: Academic Press; 1998. p 1483-8.
45. Vezzio N, Sarfati M, Yang L-P, Demeure CE, Delespesse G. Human Th2-like cell clones induce IL-12 production by dendritic cells and may express several cytokine profiles. *Int Immunol* 1996;8:1963-70.
46. Bliss J, Van Cleave V, Murray K, et al. IL-12 as an adjuvant, promotes a T helper 1 cell, but does not suppress a T helper 2 cell recall response. *J Immunol* 1996;156:887-94.
47. Wakeham J, Wang J, Magram J, et al. Lack of both types 1 and 2 cytokines, tissue inflammatory responses and immune protection during pulmonary infection by Mycobacterium bovis bacille Calmette-Guerin in IL-12-deficient mice. *J Immunol* 1998;160:6101-11.
48. Yokoi H, Kato K, Kezuka T, et al. Prevention of experimental autoimmune uveoretinitis by monoclonal antibody to interleukin-12. *Eur J Immunol* 1997;27:641-6.
49. Tarrant TK, Silver PB, Chan CC, Wiggert B, Caspi RR. Endogenous IL-12 is required for induction and expression of experimental autoimmune uveitis. *J Immunol* 1998;161:122-7.
50. Hamelmann E, Vella AT, Oshiba A, Kappler JW, Marrack P, Gelfand EW. Allergic airway sensitization induces T cell activation but not airway hyperresponsiveness in B cell-deficient mice. *Proc Natl Acad Sci U S A* 1997;94:1350-5.
51. Van der Pouw Kraan TCTM, Snijders A, Boeijs LCM, Alewijnse AE, Leurs R, Aarden LA. Histamine inhibits the production of IL-12 through interaction with H-2 receptors. *J Clin Invest* 1998;102:1866-73.

52. Elenkov IJ, Webster E, Papanicolaou DA, Fleisher TA, Chrousos GP, Wilder RL. Histamine potently suppresses human IL-12 and stimulates IL-10 production via H2 receptors. *J Immunol* 1998;161:2586-93.
53. Krouwels FH, Hol BEA, Lutter R, et al. Histamine affects interleukin-4, interleukin-5, and interferon-gamma production by human T cell clones from the airways and blood. *Am J Respir Cell Mol Biol* 1998;18:721-30.
54. Sato J, Asakura K, Murakami M, Uede T, Kataura A. Topical CTLA4-Ig suppresses ongoing mucosal immune response in presensitized murine model of allergic rhinitis. *Int Arch Allergy Immunol* 1999;119:197-204.
55. Hoshino T, Wiltout RH, Young HA. IL-18 is a potent inducer of IL-13 in NK and T cells: a new potential role for IL-18 in modulating the immune response. *J Immunol* 1999;162:5070-7.
56. Pene J, Rousset F, Briere F, et al. Interleukin 5 enhances interleukin 4-induced IgE production by normal human B cells: the role of soluble CD23 antigen. *Eur J Immunol* 1988;18:929-35.
57. Brusselle G, Kips J, Joos G, Bluethmann H, Pauwels R. Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am J Respir Cell Mol Biol* 1995;12:254-9.
58. von der Weid 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.
59. Noben-Trauth N, Kropf P, Muller I. Susceptibility to *Leishmania major* infection in interleukin-4-deficient mice. *Science* 1996;271:987-90.
60. Hogan SP, Mould A, Kikutani H, Ramsay AJ, Foster PJ. Aeroallergen-induced eosinophilic inflammation, lung damage, and airways hyperactivity in mice can occur independently of IL-4 and allergen-specific immunoglobulins. *J Clin Invest* 1997;99:1329-39.
61. Oettgen HC, Martin TR, Wynshaw-Boris A, Deng C, Drazen JM, Leder P. Active anaphylaxis in IgE-deficient mice. *Nature* 1994;370:367-70.
62. Mehlhop PD, van de Rijn M, Goldberg AB, et al. Allergen-induced bronchial hyperreactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. *Proc Natl Acad Sci U S A* 1997;94:1344-9.
63. Takeda K, Hamelmann E, Joetham A, et al. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *J Exp Med* 1997;186:449-54.

Notice to Readers

The January 2000 issue of the *Journal of Allergy and Clinical Immunology* contained several citation errors. The following articles should have carried citation lines at the end of the abstract, or in the footnotes to the article, stating the year of publication as 2000 and the volume as 105.

Ricca V, Landi M, Ferrero P, Bairo A, Tazzer C, Canonica GW, Ciprandi G. Minimal persistent inflammation is also present in patients with seasonal allergic rhinitis. 2000;105:54-7

Hanazawa T, Antuni JD, Kharitonov SA, Barnes PJ. Intranasal administration of eotaxin increases nasal eosinophils and nitric oxide in patients with allergic rhinitis. 2000;105:58-64

Goldman M, Rachmiel M, Gendler L, Katz Y. Decrease in asthma mortality rate in Israel from 1991-1995: Is it related to increased use of inhaled corticosteroids? 2000;105:71-4

Warner JA, Frederick JM, Bryant TN, Weich C, Raw GJ, Hunter C, Stephen FR, McIntyre DA, Warner JO. Mechanical ventilation and high-efficiency vacuum cleaning: A combined strategy of mite and mite allergen reduction in the control of mite-sensitive asthma. 2000;105:75-84

Agarwal SK, Marshall GD Jr. β -Adrenergic modulation of human type-1/type-2 cytokine balance. 2000;105:91-8

Macfarlane AJ, Kon OM, Smith SJ, Zeibecoglou K, Khan LN, Barata LT, McEuen AR, Buckley MG, Walls AF, Meng Q, Humbert M, Barnes NC, Robinson DS, Ying S, Kay AB. Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin. 2000;105:99-107

Shimbara A, Christodoulouopoulos P, Soussi-Gounni A, Olivenstein R, Nakamura Y, Levitt RC, Nicolaides NC, Holroyd KJ, Tsicopoulos A, Lafitte J-J, Wallaert B, Hamid QA. IL-9 and its receptor in allergic and nonallergic lung disease: Increased expression in asthma. 2000;105:108-15

Kazemi-Shirazi L, Pauli G, Purohit A, Spitzauer S, Fröschl R, Hoffman-Sommergruber K, Breiteneder H, Scheiner O, Kraft D, Valenta R. Quantitative IgE inhibition experiments with purified recombinant allergens indicate pollen-derived allergens as the sensitizing agents responsible for many forms of plant food allergy. 2000;105:116-25

Tkaczyk C, Villa I, Peronet R, David B, Chouaib S, Mécheri S. In vitro and in vivo immunostimulatory potential of bone marrow-derived mast cells on B- and T-lymphocyte activation. 2000;105:134-42

Hoshino H, Laan M, Sjöstrand M, Lötval J, Skoogh B-E, Lindén A. Increased elastase and myeloperoxidase activity associated with neutrophil recruitment by IL-17 in airways in vivo. 2000;105:143-9

Taylor AV, Swanson MC, Jones RT, Vives R, Rodriguez J, Yunginger JW, Crespo JF. Detection and quantitation of raw fish aeroallergens from an open-air fish market. 2000;105:166-9

Assa'ad A, Lierl M. Effect of acellular pertussis vaccine on the development of allergic sensitization to environmental allergens in adults. 2000;105:170-5

Berlyne GS, Efthimiadis A, Hussack P, Groves D, Dolovich J, Hargreave FE. Sputum in asthma: Color versus cell counts. 2000;105:182-3

Kurtz KM, Beatty TL, Adkinson NF Jr. Evidence for familial aggregation of immunologic drug reactions. 2000;105:184-5