Role of Interferon-γ in a Mouse Model of Allergic Conjunctivitis

Michael E. Stern,[1] *Karyn Siemasko,*[1] *Jianping Gao,*[1] *Anh Duong,*[1] *Clay Beauregard,*[2] *Virginia Calder,*[3] *and Jerry Y. Niederkorn*[2]

Purpose. To characterize the effect of repeated topical exposure to allergen in a mouse model of allergic conjunctivitis and to determine the role of interferon-γ (IFN-γ) in the pathogenesis of allergic conjunctivitis.

Methods. Wild-type BALB/c mice and IFN-γ knockout (KO) BALB/c mice were sensitized in the footpad with short ragweed (SRW) allergen and challenged topically for seven consecutive days with SRW allergen. The number of splenic $CD4^+$ Th2 cells was determined by flow cytometry, and the cytokine profile of $CD4^+$ T cells from SRW-sensitized mice was evaluated by ELISA. The role of IFN-γ in allergic conjunctivitis was also examined by timed in vivo neutralization with anti-IFN-γ antibody. Allergic conjunctivitis was evaluated clinically and histopathologically.

Results. Repeated topical challenge with SRW allergen induced allergic conjunctivitis that was characterized by lid edema, chemosis, redness, and tearing. Histopathological analysis revealed a marked conjunctival infiltrate that was predominantly neutrophils and eosinophils. IFN-γ KO mice and normal mice treated with anti-IFN-γ antibody displayed milder clinical symptoms of allergic conjunctivitis and a 70% reduction in the number of eosinophils that infiltrated the conjunctiva. Spleen cells from SRW-sensitized mice contained a large population of cells that expressed the Th2 surface marker T1/ST2 and produced IL-4, -5, and -10 and IFN-γ after stimulation with SRW allergen.

Conclusions. Repeated topical application of SRW allergen induces a form of murine allergic conjunctivitis that mimics the human counterpart. IFN-γ appears to contribute to the pathogenesis of murine allergic conjunctivitis at the effector phase, but not during the initial sensitization stage. (*Invest Ophthalmol Vis Sci.* 2005;46:3239–3246) DOI:10.1167/iovs.05-0138

Seasonal allergic conjunctivitis (SAC) is an IgE-mediated hypersensitivity disease that affects approximately 3 million people in the United States alone.[1–3] SAC is initiated by mast cell degranulation and is characterized by itching, eyelid swelling, conjunctival swelling (chemosis), and mucus deposition. This stage is often followed by an inflammatory process that culminates in an accumulation of neutrophils and eosinophils in the conjunctiva.[4] The clinical symptoms produced by mast cell degranulation and the granulocytic inflammatory responses are manifestations of Th2 immune responses.

It is widely assumed that Th1 and Th2 cells cross-regulate each other and that their cytokines are mutually inhibitory. Previous studies in a ragweed model of allergic conjunctivitis have shown that IFN-γ knockout (KO) mice and wild-type mice treated with anti-IL-12 exhibit decreased Th1 responses and have more severe conjunctival inflammatory infiltrates than do mice with intact Th1 responses.[5] A similar role for IFN-γ was found in a rat model of ovalbumin-induced conjunctivitis in which repeated administration of IFN-γ produced a 50% reduction in the eosinophil infiltrate in the conjunctivae of topically challenged rats.[6] However, there is a growing body of evidence that suggests that interactions between Th1 and Th2 immune elements are not solely antagonistic, but may in fact modulate the immune response in a much more complex manner.[7] Studies of a mouse model of allergic asthma have demonstrated that Th1 cells are needed to produce eosinophil infiltration in the lungs and adoptive transfer of allergen-specific Th1 cell–exacerbated allergic disease.[8,9] Moreover, allergen-induced airway eosinophilia in influenza-virus–infected mice is reduced in animals treated with anti-IFN-γ.[8]

The finding that Th1 and Th2 cells can contribute to the pathophysiology of some types of allergic reactions prompted us to consider the role of the Th1 cytokine, IFN-γ, in a mouse model of chronic allergic conjunctivitis. Previous models of allergic conjunctivitis have used a single topical challenge with allergen and have not considered the role of repeated exposure to allergens, as occurs in seasonal allergic conjunctivitis. Therefore, the present study also examined the effect of repeated ocular exposure to allergens on the pathophysiology of allergic conjunctivitis.

Materials and Methods

Animals

Animal studies were approved by the Institutional Review Board of the University of Texas Southwestern Medical Center at Dallas and the Allergan Animal Care and Use Committee. Animals were housed and cared for in accordance with the guidelines of the University Committee for the Humane Care of Laboratory Animals, National Institutes of Health, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Female BALB/c ($H\text{-}2^d$; 5–9 weeks old) were obtained from Taconic (Germantown, NY) or The Jackson Laboratory (Bar Harbor, ME). Female IFN-γ knockout (KO) mice ($C.129S7(B6)\text{-}Ifng^{tm1Ts/J}$, formerly $BALB/c\text{-}Ifng^{tm1Ts}$) were purchased from The Jackson Laboratory.

Induction of Atopic Conjunctivitis

The protocol used to sensitize and challenge mice was modified from Magone et al.[10] BALB/c mice were immunized with 50 μg of short ragweed (SRW) pollen (from *Ambrosia artemisiifolia*; International Biologicals, Piedmont, OK) in 5 mg alum (Imject; Pierce Biotechnology, Rockford, IL) by footpad injection on day 0. Allergic conjunctivitis was induced by a multihit topical challenge method, in which immunized mice were given topical 1.5-mg applications of SRW pollen in 10

From [1]Allergan, Irvine, California; the [2]Department of Ophthalmology, University of Texas Southwestern Medical Center, Dallas, Texas; and the [3]Department of Ophthalmology, University College London, London, United Kingdom.

Supported entirely by Allergan.

Submitted for publication February 2, 2005; revised April 26 and May 11, 2005; accepted June 24, 2005.

Disclosure: **M.E. Stern**, Allergan (E, F); **K. Siemasko**, Allergan (E, F); **J. Gao**, Allergan (E, F); **A. Duong**, Allergan (F); **C. Beauregard**, Allergan (F); **V. Calder**, Allergan (F); **J.Y. Niederkorn**, Allergan (F)

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Jerry Y. Niederkorn, Department of Ophthalmology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-9057; jerry.niederkorn@utsouthwestern.edu.

μL PBS in the right eye once a day from days 10 to 16. Animals were examined clinically for signs of immediate hypersensitivity responses 20 minutes after each topical challenge with SRW pollen. A clinical scoring scheme, similar to that described by Magone et al.,[10] was used that evaluated chemosis, conjunctival redness, lid edema, and tearing. Each parameter was graded on a scale ranging from 0 to 4+. Where indicated, BALB/c wild-type mice received intraperitoneal injections of 500 μg of anti-IFN-γ antibody (R4-6A2; American Type Culture Collection, Manassas, VA) or rat IgG (Sigma-Aldrich, St. Louis, MO). Eosinophil and neutrophil cells were counted by two masked observers in five different sections of the conjunctival forniceal area.

In Vivo Antibody Treatment

Wild-type BALB/c mice were treated with intraperitoneal injections of anti-IFN-γ antibody (500 μg/dose; catalog no. HB170; R4-6A2 hybridoma; rat IgG anti-mouse IFN-γ; American Type Culture Collection) before, during, or after topical challenge with SRW pollen, which was always initiated 10 days after footpad immunization. Anti-IFN-γ antibody was administered 5 days before and 5 days after the initial footpad sensitization with SRW and alum. A second group of mice was treated with intraperitoneal injections of anti-IFN-γ antibody 5 and 11 days after footpad immunization (i.e., bracketing the initiation of topical challenge). A third group of mice was treated 11 and 16 days after footpad sensitization (i.e., after the initiation of topical challenge). All mice were challenged topically with SRW allergen 10 days after the footpad immunization with SRW allergen and alum. Daily topical challenge with SRW was continued for a total of seven consecutive days.

Allergen-Specific Cytokine Stimulation

On day 17 of the multihit induction of conjunctivitis, mice were killed and the spleens were removed. Splenic $CD4^+$ T cells were isolated using a magnetic microbead system (MACS; Miltenyi Biotec, Auburn, CA), as previously described.[1] Enriched $CD4^+$ cells were incubated with freshly isolated BALB/c splenic dendritic cells and short ragweed pollen soluble extract (Greer Laboratories, Lenoir, NC) for 48 hours. Non–antigen-specific T-cell stimulation was performed with immobilized anti-CD3 (BD-Pharmingen, San Diego, CA) and soluble anti-CD28 (BD-Pharmingen) for 48 hours. Six hours before harvest, 1 μg/mL ionomycin (Sigma-Aldrich) and 25 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) were added to each well to stimulate cytokine release. ELISAs for mouse IL-2, -4, -5, and -10, and IFN-γ were performed on culture supernatants according to the manufacturer's instructions (Quantikine ELISA kits; R&D Systems, Minneapolis, MN).

As a positive control for Th1 cytokine production, naïve BALB/c mice were immunized subcutaneously with 200 μL complete Freund's adjuvant (CFA; Sigma-Aldrich) 14 days before the spleens were harvested. As a positive control for Th2 cytokine production, naïve BALB/c mice were immunized intraperitoneally with 500 μg aqueous extract of *Leishmania major* (kindly provided by George L. Stewart, University of Western Florida, Pensacola, FL) 21, 14, and 7 days before the spleens were harvested.

Flow Cytometric Analysis

In vitro–stimulated $CD4^+$ T cells were harvested and fixed in 2% buffered formalin. Cells were stained with 1 μg/mL rat anti-mouse T1/ST2-FITC (BD Biosciences, Franklin Lakes, NJ) or 1 μg/mL FITC rat IgG isotype control (BD-Pharmingen) for 30 minutes at 4°C. FITC staining was detected on a flow cytometer (FACScalibur; BD Biosciences), with 1×10^4 events per sample acquired for analysis.

SRW-Specific IgE ELISA

SRW extract of surface antigens was purchased from Greer Laboratories (Lenoir, NC) and biotinylated with a kit (Pierce Biotechnology). Antibody specific to the Fc portion of mouse IgE (OpTEIA Mouse IgE Kit; BD Biosciences) was used to coat ELISA plates (96-well; Nalge Nunc International, Rochester, NY) as outlined in the manufacturer's protocol. Because IgE specific to SRW allergens was not commercially available, normal mouse IgE (concentrations ranging from 1.6 to 100 ng/mL) was used as the standard for calibrating the ELISA results (BD Biosciences). Anti-IgE was used as the secondary antibody, in place of biotinylated SRW extract, for establishing the standards. Plates were read using a plate reader (Softmax Pro; Molecular Devices, Sunnyvale, CA) at the absorbance wavelength of 450 nm.

Immunohistochemistry

The cryosections (10 μm) from mouse globes were baked at 37°C overnight. Sections were postfixed with cold acetone (4°C) for 3 minutes and air dried for 30 minutes. Samples were washed with PBS five times (3 minutes per wash). They were blocked with 4% BSA-PBS at room temperature for 2 hours and 4% normal donkey serum for 1 hour followed by overnight incubation at 4°C in a humidified chamber with anti-vascular endothelial cell adhesion molecule (VCAM)-1 (BD Pharmingen, 31.25 μg/mL used at a 1:25 dilution); von Willebrand Factor (1:100 dilution, no. AB7356; Chemicon, Temecula, CA), or normal rat IgG2a (BD Pharmingen). The secondary antibodies (Alexa 488 FITC, no. A-21208 and/or Alexa 568 TRITC, no. A-11011; Molecular Probes, Eugene, OR) were applied and incubated with the samples for 1 hour at room temperature.

Statistics

Student's *t*-test was used to evaluate statistical significance in the various experiments. $P < 0.05$ was considered significant. Differences at the level of $P < 0.005$ were recorded as <0.005 and not as the exact probability (e.g., $P = 0.000005$ was recorded as $P < 0.005$).

Results

Effect of Repeated Exposure to Topically Applied Allergen on Prolonged Allergic Conjunctivitis and Inflammation

To mimic repeated exposure to allergens, SRW allergen-sensitized mice were challenged with daily topical applications of SRW allergen for seven consecutive days. Clinical observations revealed typical symptoms of allergic conjunctivitis that were detected within 20 minutes of topical challenge with SRW allergen and persisted until the next challenge 24 hours later (Fig. 1A). Histopathological analysis demonstrated intense inflammation of the conjunctivae (Fig. 1B). Neutrophils and eosinophils were the predominant inflammatory cells in the affected conjunctivae and were present in specimens collected at 24, 48, and 72 hours after the seventh topical challenge (Fig. 1C).

Splenic $CD4^+$ T cells from mice sensitized in the footpad and topically challenged with SRW allergen were cultured in vitro with SRW allergen and the percentage of Th2 cells was determined by flow cytometry using the Th2-specific surface marker, T1/ST2.[11] The results demonstrate that SRW-sensitized mice displayed approximately a fourfold higher number of SRW-responsive Th2 cells than did either the unsensitized controls or mice sensitized with the Th1 immunogen, CFA (Fig. 2). ELISA analysis of supernatants of $CD4^+$ T cells cultured in the presence of SRW allergen revealed a steep increase in the synthesis of Th2 cytokines IL-4, -5, and -10 (Fig. 3A). The enhanced production of Th2 cytokines by $CD4^+$ T cells from SRW-sensitized mice did not inhibit the production of IFN-γ, which was of the same magnitude as naïve control mice and cells from mice sensitized with the Th1 immunogen CFA (Fig. 3B).

Allergic Conjunctivitis in IFN-γ KO Mice

The simultaneous production of IL-4, -5, and -10 and IFN-γ by $CD4^+$ T cells after in vitro stimulation with SRW allergen suggested that strict polarization of the T-helper cell response did not occur in this model of allergic conjunctivitis. Accordingly, experiments were performed in IFN-γ KO mice, to as-

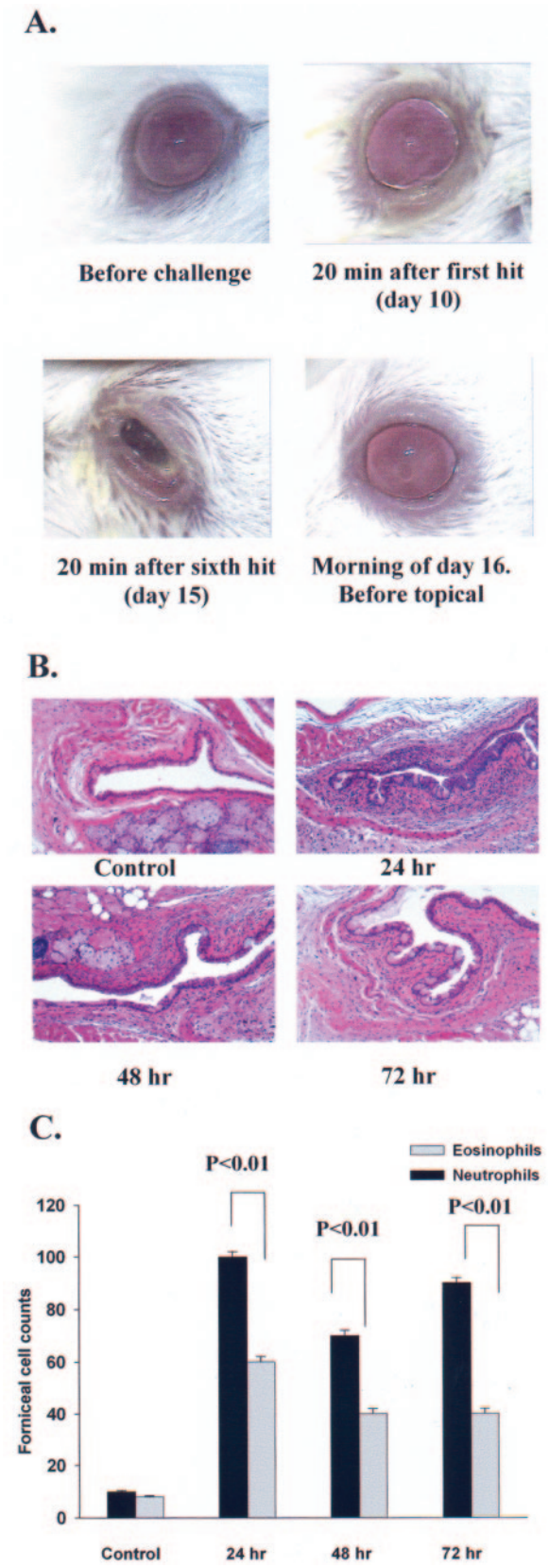

FIGURE 1. Allergic conjunctivitis induced by repeated topical application of SRW allergen. (**A**) Clinical appearance of eyes after topical challenge with SRW pollen. (**B**) Histopathological features of conjunctivae after topical challenge with SRW allergen. Original magnification, ×20. (**C**) Neutrophils and eosinophils were enumerated in the conjunctivae collected after the seventh topical application of SRW allergen ($n = 5$). Forniceal cell counts are represented as the mean ± SEM number of cells/five high-power fields.

certain the role of Th1 cytokine expression in allergic conjunctivitis. Although wild-type mice developed allergic conjunctivitis that paralleled the previous experiments, IFN-γ KO mice had greatly reduced clinical signs of allergic conjunctivitis (Fig. 4A). Chemosis, lid edema, redness, and tearing scores were significantly reduced in IFN-γ KO mice (Fig. 4B). Moreover, the number of infiltrating neutrophils and eosinophils was reduced by approximately 66% and 78%, respectively, in the IFN-γ KO hosts (Fig. 5A). These results emphasize that in the absence of IFN-γ, both the clinical and pathologic phenotypes of allergic conjunctivitis are significantly reduced. The reduction in neutrophils and eosinophils in the affected conjunctivae in IFN-γ KO mice was not, because of an overall depression in the quantity of circulating granulocytes, as peripheral blood cell counts revealed a similar number of neutrophils in both mouse strains and even a significant increase in the number of eosinophils in IFN-γ KO mice (Fig. 5B). SRW sensitized and topically challenged mice exhibited 30-fold (BALB/c WT) and 16-fold (IFN-γ KO) higher SRW-specific IgE antibody titers compared with the nontreated control mice (data not shown). The IFN-γ KO mice displayed lower anti-SRW allergen IgE antibody titers compared with SRW sensitized and challenged wild-type BALB/c mice. However, IFN-γ KO mice produced sufficient SRW-specific IgE (0.85 μg/mL ± 0.04) to suggest that they were not completely inhibited from expressing Th2-type responses.

Effect of IFN-γ Depletion on Allergic Conjunctivitis

Results from experiments using KO mice should be interpreted with caution, as KO mice often possess redundant mechanisms that compensate for the deleted gene function. Moreover, KO mice sometimes express unanticipated phenotypic changes that are not directly attributed to the gene that has been deleted.[12,13] Therefore, additional experiments were performed in wild-type BALB/c mice that were treated with intraperitoneal injections of anti-IFN-γ antibody, given before, during, or after topical challenge with SRW pollen. Administration of anti-IFN-γ antibody at times surrounding the initial footpad

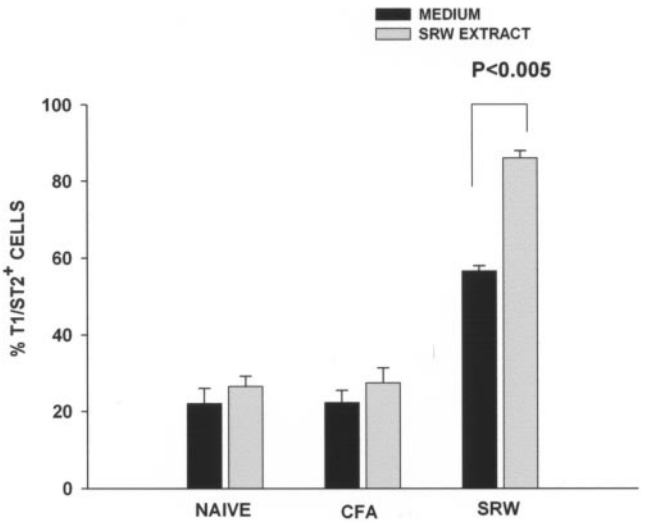

FIGURE 2. Percentage of splenic $CD4^+$ T cells expressing T1/ST2 surface antigen in mice challenged topically with SRW pollen. Splenic $CD4^+$ T cells were cultured in vitro with SRW pollen extract and examined by flow cytometry for the expression of Th2 surface marker, T1/ST2. $CD4^+$ T cells from mice sensitized by subcutaneous injection with the Th1 immunogen and complete Freund's adjuvant (CFA) were similarly processed and tested for expression of T1/ST2. Results are expressed as the mean ± SEM.

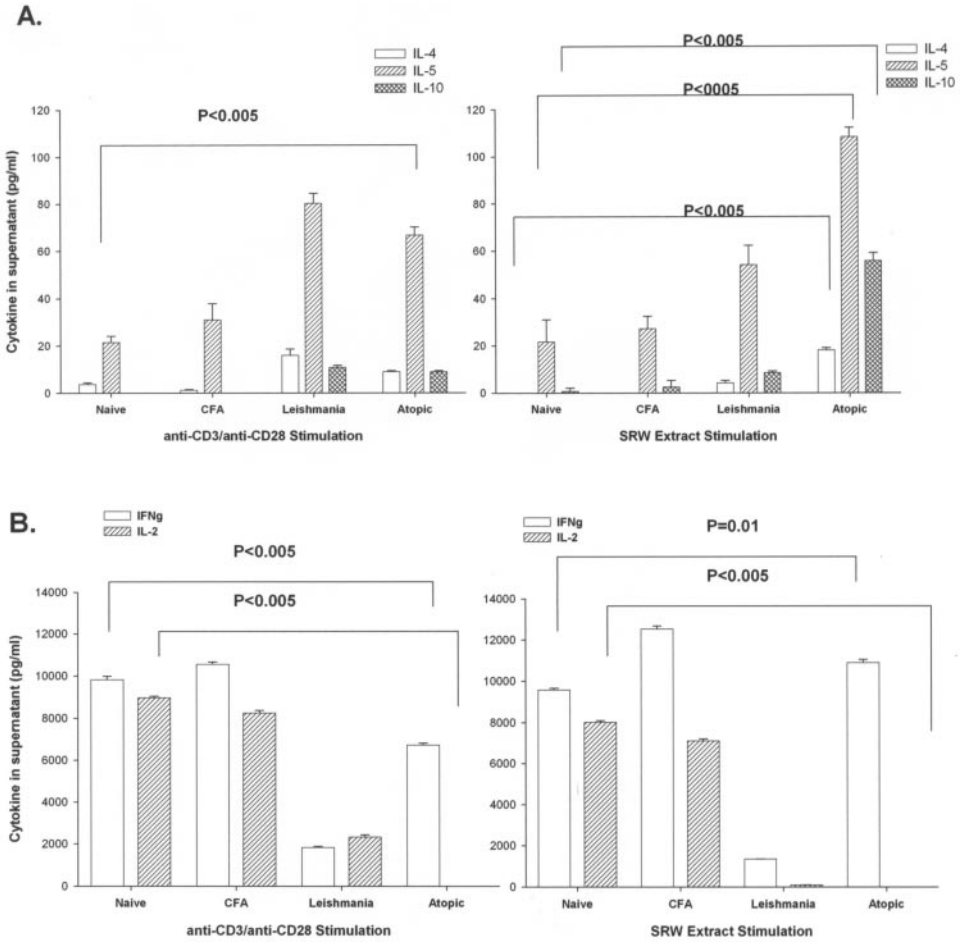

FIGURE 3. Th1 and Th2 cytokine production by $CD4^+$ T cells from atopic mice. Splenic $CD4^+$ T cells were cultured in the presence of either SRW pollen extract or plate-bound anti-CD3 and soluble anti-CD28 antibodies. Supernatants were evaluated for Th1 and Th2 cytokines using a conventional capture ELISA. (**A**) IL-4, -5, and -10. (**B**) IL-2 and IFN-γ. Controls consisted of naïve mice, mice immunized subcutaneously with CFA, or mice immunized intraperitoneally with *Leishmania* antigen. Results are expressed as the mean ± SEM.

sensitization with SRW and alum, but before topical challenge, resulted in a significant increase in the number of neutrophils that infiltrated the conjunctiva (Fig. 6). By contrast, delaying anti-IFN-γ antibody treatment until 5 or 11 days after initial sensitization mitigated both the clinical manifestations of allergic conjunctivitis and produced a 50% and 75% reduction in the number of infiltrating eosinophils respectively (Fig. 6). Additional experiments confirmed the mitigating effects of anti-IFN-γ antibody by comparing the effects of anti-IFN-γ antibody with an isotype control antibody. As before, intraperitoneal injection of anti-IFN-γ antibody, given either as two doses on days 1 and 6 (i.e., days 11 and 16 after footpad sensitization) of topical challenge or given continuously beginning on the day of topical challenge (i.e., day 10) and continuing through day 17, produced significant reductions in the numbers of neutrophils and eosinophils that infiltrated the affected conjunctivae (Fig. 7). Maximum mitigation of conjunctival inflammation occurred when anti-IFN-γ was given throughout the entire period of topical challenge with SRW allergen (Fig. 7A). By contrast, treatment with the control irrelevant rat IgG had no inhibitory effect on the infiltration of neutrophils and eosinophils into the conjunctiva (Fig. 7B).

IFN-γ has pleiotropic effects on the immune system including the regulation of VCAM-1 expression on vascular endothelium.[14] VCAM-1 is an important cell adhesion molecule that binds to its coligand, VLA-4, and facilitates extravasation of eosinophils.[15,16] Accordingly, VCAM-1 expression on conjunctival vascular endothelium of SRW-sensitized and -challenged mice was assessed by immunohistochemistry. As expected, VCAM-1 was detected on the conjunctival vascular endothelium of wild-type BALB/c mice sensitized with SRW allergen, but was conspicuously absent in similarly treated IFN-γ KO mice (Fig. 8).

DISCUSSION

Seasonal allergic conjunctivitis is characterized by mast cell degranulation and release of vasoactive amines, which provoke itching, eyelid swelling, conjunctival swelling, and mucus secretion. This is often followed by an inflammatory response involving neutrophils and eosinophils. To date, therapies for allergic conjunctivitis have focused on the mast cell phase of the conjunctival allergic response and have used agents that either prevent mast cell degranulation or block vasoactive amines that are released from mast cells. The present study was designed to explore the immunologic and inflammatory underpinnings of this syndrome and to establish an animal model of allergic conjunctivitis that mimicked the human counterpart. This was accomplished through chronic ocular exposure to SRW allergens, which resulted in the development of late-phase conjunctival inflammation. The present model fits this description and displays parameters indicative of a Th2 allergic conjunctivitis including: (1) clinical and histopathologic features that mimic allergic conjunctivitis; (2) repeated ocular exposure to allergen, which is believed to occur in human SAC; (3) preferential expansion of T cells expressing the Th2 surface marker, T1/ST2; and (4) enhanced production of IL-4 and -5 by Th2 cells after stimulation with SRW allergen. However, the results revealed an unexpected finding indicating that the elaboration of IFN-γ by $CD4^+$ T cells from SRW-sensitized mice.

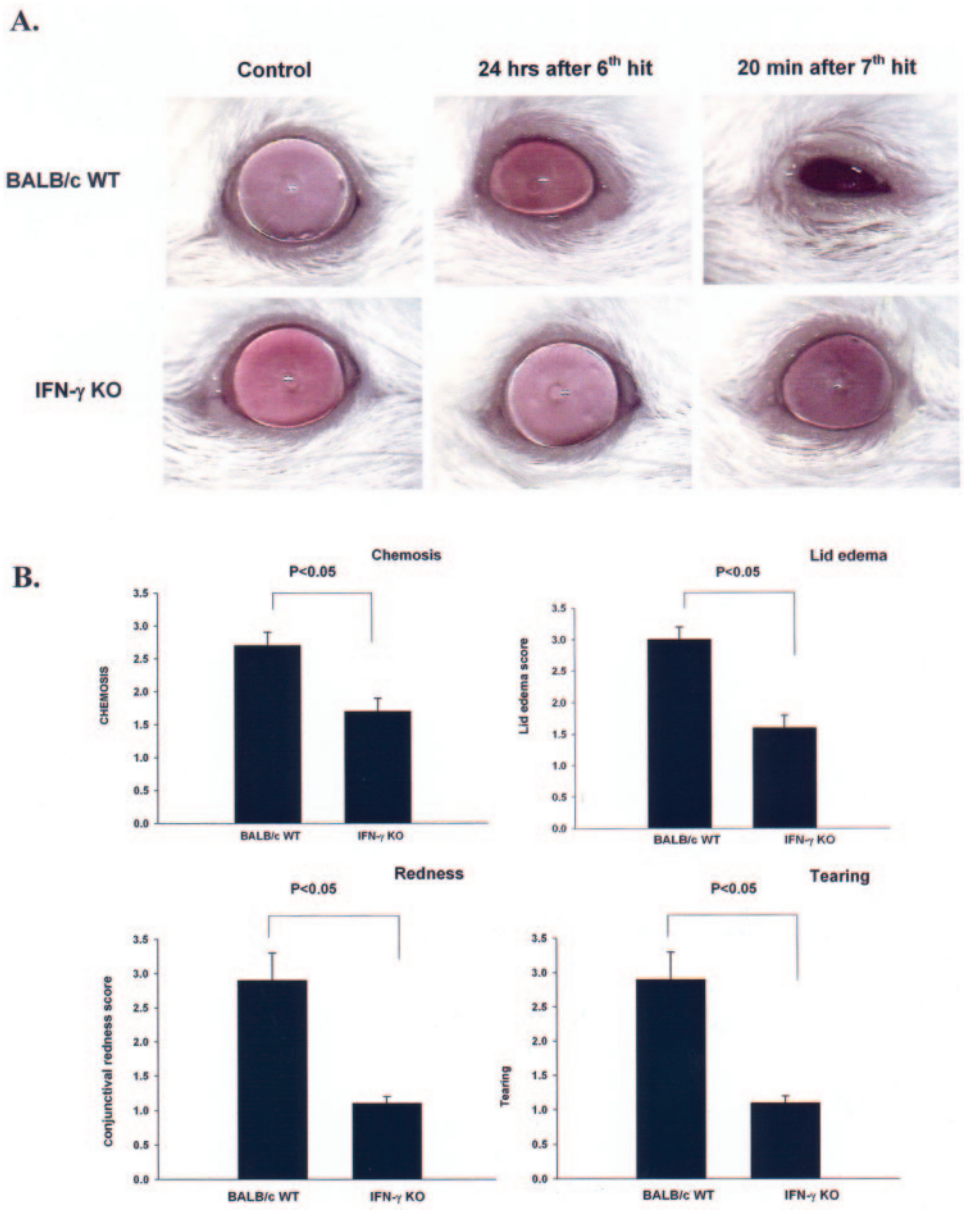

FIGURE 4. Allergic conjunctivitis in IFN-γ KO mice and wild-type (WT) mice. Mice were immunized in the footpads with SRW allergen and alum and challenged topically with SRW allergen for seven consecutive days. (**A**) Clinical appearance of eyes 24 hours after the sixth and seventh topical challenges with SRW allergen. (**B**) Clinical scores 20 minutes after the seventh topical challenge with SRW pollen.

The production of IFN-γ in the face of an expanded SRW allergen-specific Th2 cell population and elevated Th2 cytokines suggested that polarization of Th2 responses was incomplete and allowed the production of a significant quantity of IFN-γ. The production of IFN-γ may be an integral component of allergic conjunctivitis, as has been suggested in some Th2 diseases.[7–9,17] This hypothesis was tested by inducing allergic conjunctivitis in mice that were unable to produce IFN-γ. Although it was possible to induce allergic conjunctivitis in IFN-γ KO hosts, there was a significant diminution in the clinical scores and a 66% reduction in neutrophils and a 78% reduction in eosinophils in the conjunctiva. The reduction of inflammatory cells was not due to a diminished number of circulating neutrophils and eosinophils in the IFN-γ KO mice, as the neutrophil and eosinophil cell counts in the peripheral blood were the same in wild-type and IFN-γ KO mice. Timed administration of anti-IFN-γ antibody into wild-type mice revealed that neutralization of IFN-γ during the sensitization phase of the immune response to SRW resulted in more severe allergic conjunctivitis, as might be expected since IFN-γ is known to have an inhibitory effect on the generation of Th2 responses. By contrast, administration of anti-IFN-γ after the initiation of the primary immune response and at a time when the commitment to a Th2 pathway had already been made, resulted in a mitigation in allergic conjunctivitis. These results may be indicative of the pleiotropic effects of IFN-γ. IFN-γ may play a role as a "vascular gatekeeper" in the inflammatory immune response by modulating the expression of cell adhesion molecules on the conjunctival vascular endothelium.

Our results differ from previous findings reported by Magone et al.,[5] who found that SRW allergen-induced allergic conjunctivitis is more severe in IFN-γ KO mice than in wild-type control animals and concluded that IFN-γ has a protective effect on the late-phase cellular infiltration in allergic conjunctivitis. Several explanations may account for the discordance in our findings and those of Magone et al. The IFN-γ KO mice in our study were on the Th2-prone BALB/c background, whereas those used by Magone et al. were on the background of the C57BL/6 mouse, which is known to be a Th1-prone mouse strain. Moreover, ELISA analysis of serum from BALB/c IFN-γ KO mice revealed the presence of low levels of IFN-γ (data not shown). It is possible that a similar condition exists in IFN-γ KO mice on the C57BL/6 background. Finally, our model involves repeated daily ocular challenge with SRW allergens,

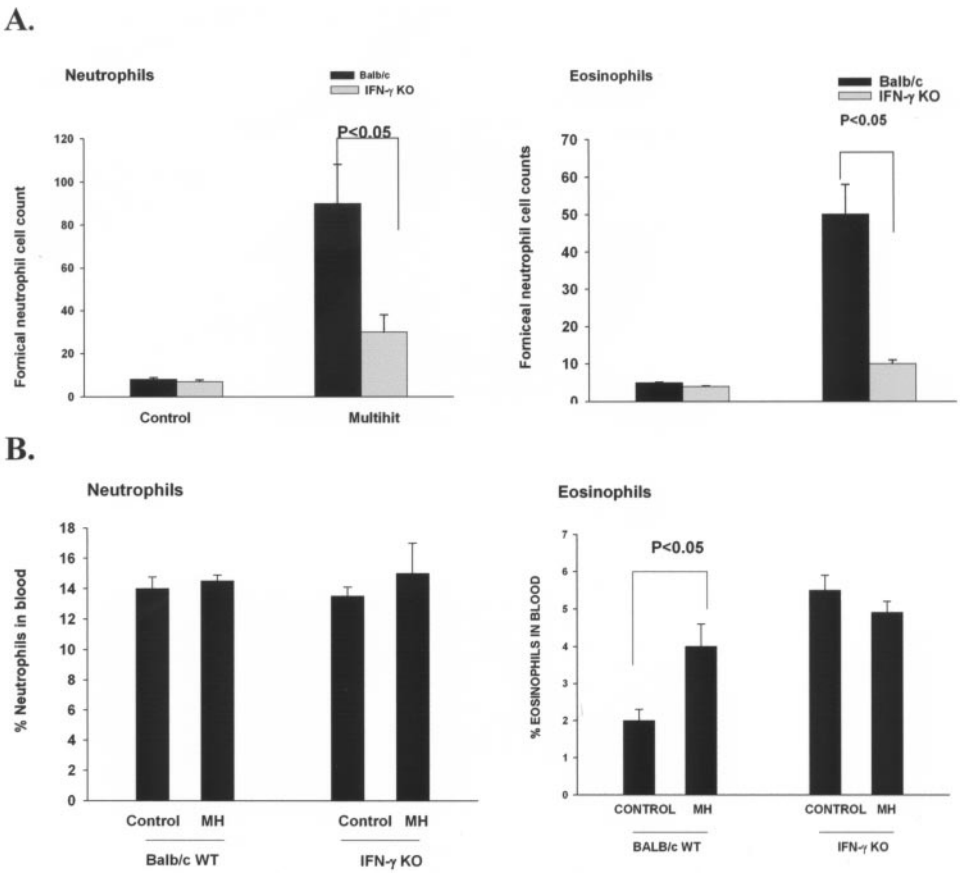

FIGURE 5. Neutrophil and eosinophil profiles in IFN-γ KO mice and wild-type mice expressing allergic conjunctivitis. (**A**) Number of neutrophils and eosinophils infiltrating the fornix 24 hours after the seventh topical challenge with SRW allergen. Forniceal cell counts are represented as the number of cells/five high-power fields. (**B**) Number of neutrophils and eosinophils in peripheral blood. Peripheral blood was examined 24 hours after the seventh topical challenge. Results are expressed as mean ± SEM. MH, multihit topical challenge with SRW allergen.

whereas Magone et al. used a single ocular challenge with SRW allergens. It is interesting that eosinophil infiltration was markedly reduced in IL-12 KO mice used in the study by Magone et al. IL-12 is known to promote IFN-γ production, and it is possible that the mitigation of allergic conjunctivitis in IL-12 KO mice is due to a downregulation in IFN-γ.

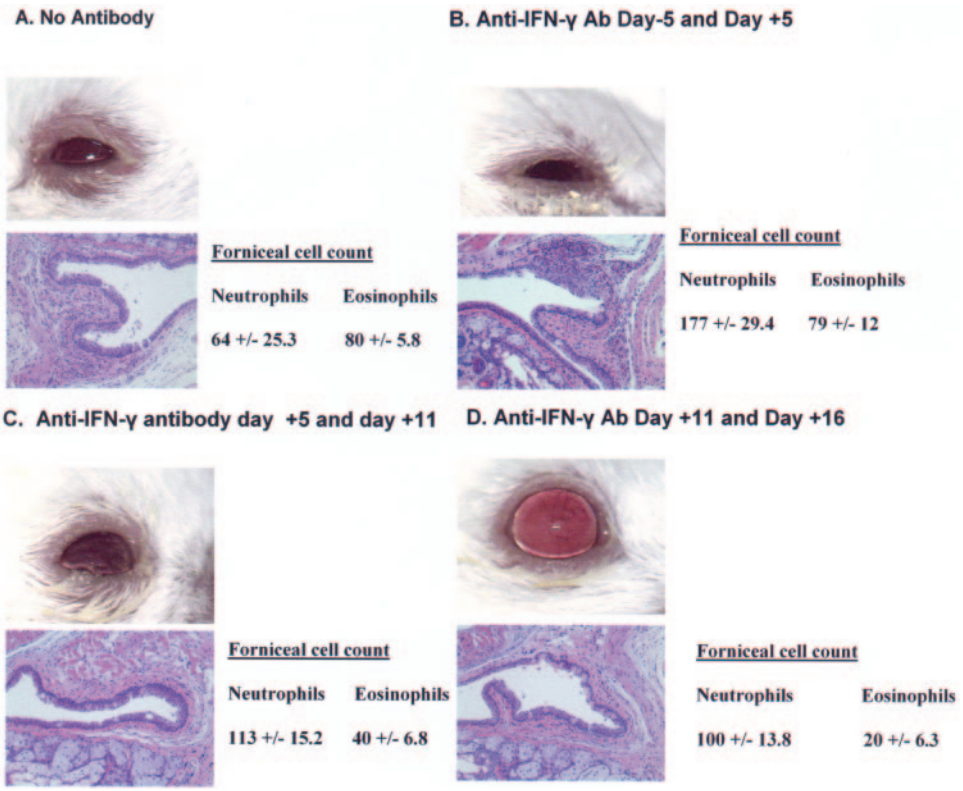

FIGURE 6. Effect of anti-IFN-γ antibody treatment on the development of allergic conjunctivitis. All BALB/c mice were immunized with SRW allergen and alum and challenged topically with SRW allergen for seven consecutive days beginning 10 days after initial subcutaneous immunization. (**A**) No antibody treatment; (**B**) anti-IFN-γ antibody was administered 5 days before and 5 days after initial subcutaneous immunization with SRW and alum; (**C**) anti-IFN-γ antibody administered 5 and 11 days after initial subcutaneous immunization with SRW and alum; and (**D**) anti-IFN-γ antibody administered 11 and 16 days after initial subcutaneous immunization with SRW and alum. All photographs were taken 10 minutes after the seventh topical challenge with SRW allergen. Magnification, ×20.

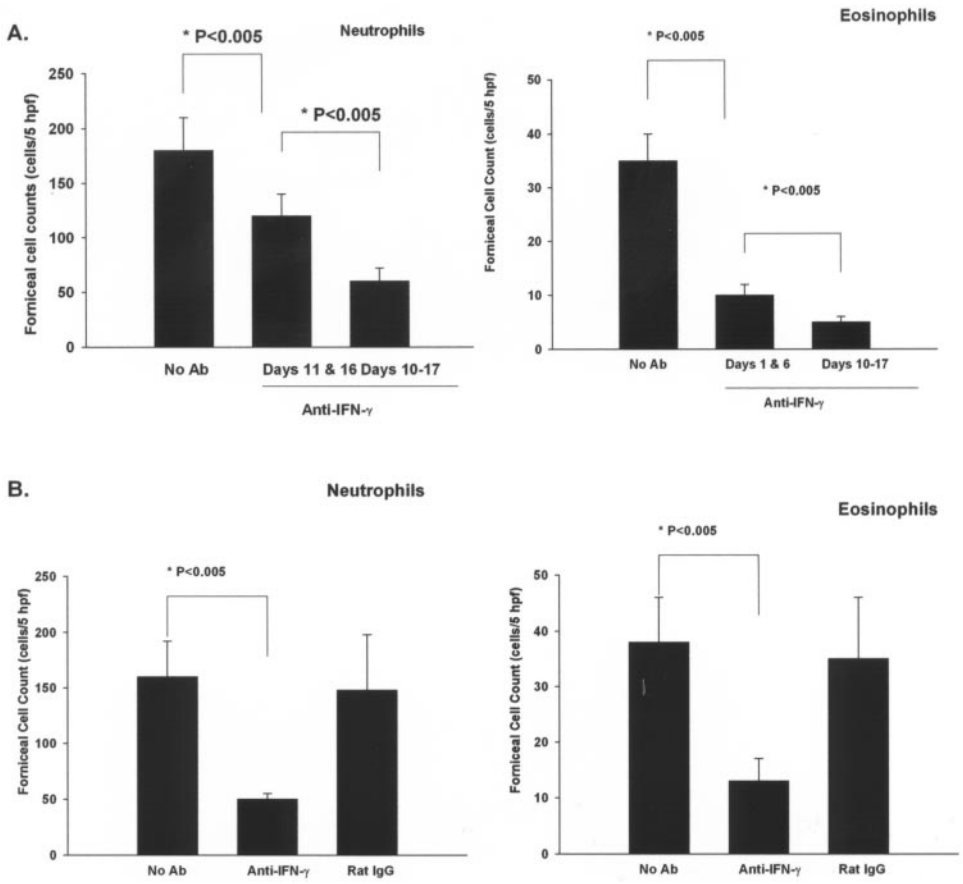

FIGURE 7. Effect of anti-IFN-γ antibody treatment given for the entire SRW topical challenge period. Atopic mice received intraperitoneal injections of (**A**) anti-IFN-γ on days 11 and 16 or days 10 to 17, or (**B**) anti-IFN-γ or rat IgG serum on days 10 to 17. Cell counts are reported as number of cells/five high-powered fields (hpf).

Our results with IFN-γ KO mice and wild-type mice treated with anti-IFN-γ antibody clearly demonstrate that IFN-γ is essential for the full expression of allergic conjunctivitis. However, the mechanisms for this effect remain a mystery. Administration of anti-IFN-γ antibody during the induction phase of the immune response exacerbated allergic conjunctivitis and is in keeping with the known cross-regulatory effects of IFN-γ on Th2 cytokines. By contrast, administration of anti-IFN-γ antibody at a time when a Th2 immune response was well under way produced a steep reduction in the number of eosinophils that infiltrated the conjunctivae. This effect was not simply due to a reduction in the number of circulating eosinophils in the IFN-γ KO mice, as wild-type mice and IFN-γ KO mice displayed a similar number of eosinophils in their peripheral blood. IFN-γ is a pleiotropic cytokine that affects many cell processes, including the upregulation of VCAM-1.[14] VCAM-1 is an important cell adhesion molecule, which binds to its coligand, VLA-4, and facilitates extravasation of eosinophils.[15,16] IFN-γ has been shown to upregulate the expression of VCAM-expression on vascular endothelium[14]; and, as shown in the current study, there is a significant diminution of VCAM-1 expression on the conjunctival vascular endothelium in IFN-γ KO mice. Moreover, it is noteworthy that VCAM-1 expression is upregulated in the conjunctivae in chronic forms of allergic conjunctivitis, such as vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC)[18]; and, in AKC, there is evidence of increased expression of IFN-γ by $CD3^+$ T cells within the conjunctivae.[19] Thus, IFN-γ may contribute to the pathogenesis of allergic conjunctivitis by regulating the expression of VCAM-1 and thereby facilitating extravasation of circulating eosinophils into the allergic conjunctiva.

The present findings add to a growing body of evidence that both Th1 and Th2 cytokines are needed for the development of allergic inflammatory responses.[7–9,17,20] The Th1 cytokine

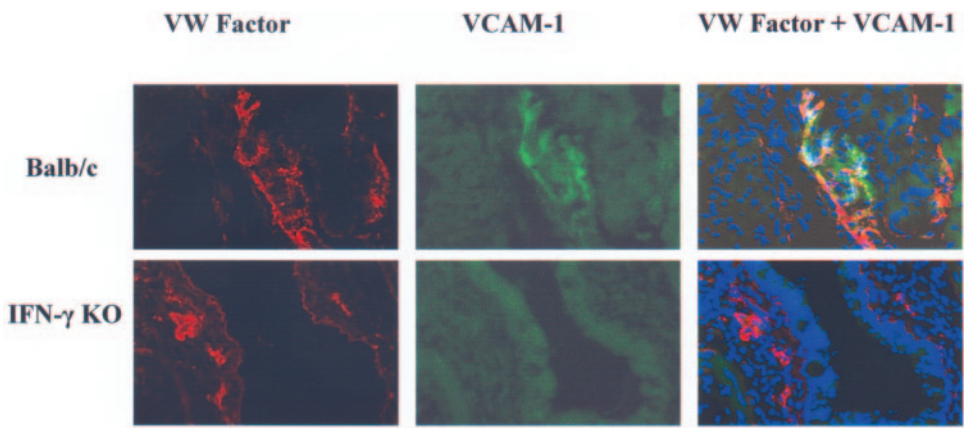

FIGURE 8. Expression of VCAM-1 on conjunctival blood vessels in wild-type and IFN-γ KO mice. Wild-type and IFN-γ KO BALB/c mice were immunized with SRW allergen and alum and challenged topically with SRW allergen for seven consecutive days beginning 10 days after initial subcutaneous immunization. Conjunctivae were stained with anti-von Willebrand factor (VW Factor; *red*) and anti-VCAM-1 (*green*) monoclonal antibodies. Cell nuclei were stained with DAPI (*blue*). The staining shown is representative of that found in three of the 4 IFN-γ KO mice and four of four wild-type BALB/c mice tested.

IFN-γ plays a major role in regulating the infiltration of eosinophils into the allergic conjunctiva in the mouse and, as such, is a potential therapeutic target for the management of chronic allergic conjunctivitis. However, it remains to be determined whether IFN-γ plays a similar role in the pathophysiology of allergic conjunctivitis in humans.

References

1. Foster CS. The pathophysiology of ocular allergy: current thinking. *Allergy*. 1995;50:6–9.
2. Ehlers WH, Donshik PC. Allergic ocular disorders: a spectrum of diseases. *CLAO J*. 1992;18:117–124.
3. Trocme SD, Raizman MB, Bartley GB. Medical therapy for ocular allergy. *Mayo Clin Proc*. 1992;67:557–565.
4. Reiss JAM, George MA, Wedner HJ. Allergic conjunctivitis. In: Pepose JS, Holland GN, Wilhemus KR, eds. *Ocular Infection and Immunity*. Mosby: St. Louis; 1996:345–358.
5. Magone MT, Whitcup SM, Fukushima A, et al. The role of IL-12 in the induction of late-phase cellular infiltration in a murine model of allergic conjunctivitis. *J Allergy Clin Immunol*. 2000;105:299–308.
6. Fukushima A, Fukata K, Ozaki A, et al. Exertion of the suppressive effects of IFN-gamma on experimental immune mediated blepharoconjunctivitis in Brown Norway rats during the induction phase but not the effector phase. *Br J Ophthalmol*. 2002;86:1166–1171.
7. Gor DO, Rose NR, Greenspan NS. TH1-TH2: a procrustean paradigm. *Nat Immunol*. 2003;4:503–505.
8. Dahl ME, Dabbagh K, Liggitt D, Kim S, Lewis DB. Viral-induced T helper type 1 responses enhance allergic disease by effects on lung dendritic cells. *Nat Immunol*. 2004;5:337–343.
9. Randolph D A, Stephens R, Carruthers CJ, Chaplin DD. Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation. *J Clin Invest*. 1999;104:1021–1029.
10. Magone MT, Chan CC, Rizzo LV, Kozhich AT, Whitcup SM. A novel murine model of allergic conjunctivitis. *Clin Immunol Immunopathol*. 1998;87:75–84.
11. Monney L, Sabatos CA, Gaglia JL, et al. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature*. 2002;415:536–541.
12. Hooper C. Genetic knockouts: surprises, lessons, and dreams. *J NIH Res*. 1992;4:34–35.
13. Golovkina TV, Shlomchik M, Hannum L, Chervonsky A. Organogenic role of B lymphocytes in mucosal immunity. *Science*. 1999; 286:1965–1968.
14. Parr MB, Parr EL. Interferon-gamma up-regulates intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 and recruits lymphocytes into the vagina of immune mice challenged with herpes simplex virus-2. *Immunology*. 2000;99:540–545.
15. Sagara H, Matsuda H, Wada N, et al. A monoclonal antibody against very late activation antigen-4 inhibits eosinophil accumulation and late asthmatic response in a guinea pig model of asthma. *Int Arch Allergy Immunol*. 1997;112:287–294.
16. Weg VB, Williams TJ, Lobb RR, Nourshargh S. A monoclonal antibody recognizing very late activation antigen-4 inhibits eosinophil accumulation in vivo. *J Exp Med*. 1993;177:561–566.
17. Magnan AO, Mely LG, Camilla CA, et al. Assessment of the Th1/Th2 paradigm in whole blood in atopy and asthma. Increased IFN-gamma-producing CD8(+) T cells in asthma. *Am J Respir Crit Care Med*. 2000;161:1790–1796.
18. Bacon AS, McGill JI, Anderson DF, et al. Adhesion molecules and relationship to leukocyte levels in allergic eye disease. *Invest Ophthalmol Vis Sci*. 1998;39:322–330.
19. Metz DP, Hingorani M, Calder VL, Buckley RJ, Lightman SL. T-cell cytokines in chronic allergic eye disease. *J Allergy Clin Immunol*. 1997;100:817–824.
20. Krug N, Madden J, Redington AE, et al. T-cell cytokine profile evaluated at the single cell level in BAL and blood in allergic asthma. *Am J Respir Cell Mol Biol*. 1996;14:319–326.