

Regulatory Function of CpG-Activated B Cells in Late-Phase Experimental Allergic Conjunctivitis

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PURPOSE. To determine how CpG, an immunostimulatory sequence, affects experimental allergic conjunctivitis and to determine the mechanisms of its action.

METHODS. Experimental allergic conjunctivitis was induced in mice to investigate the suppressive mechanism of CpG treatment. Cytokine profiling, fluorescence-activated cell sorting analyses, and adoptive transfer were used to analyze suppressive mechanisms after CpG treatment.

RESULTS. Administration of the CpG oligonucleotide induced significant splenomegaly. Adoptive transfer of the splenocytes isolated from CpG-treated mice was able to confer resistance to allergen-induced inflammatory responses in recipient mice. CpG treatment led to a transient upregulation of IL-1ra, IL-18, IL-1α, and IL-12 in the spleen, draining lymph nodes, and conjunctiva. In contrast, IL-10 showed a marked and sustained induction in the inductive and effector tissues. Splenomegaly after CpG exposure was reduced in IL-10-deficient mice, indicating that IL-10 is required for immune remodeling of the spleen. Analyses of allergen-sensitized mice deficient in IL-10 exacerbated the late-phase inflammatory responses. Fluorescence-activated cell sorting analysis of the CpG-induced splenocyte subsets showed that the predominant source of IL-10 was B220⁺ CD19⁺ CD23⁺ IgM⁺ CD40⁺ class II^{high} follicular B cells. Adoptive transfer of IL-10-deficient B cells exacerbated eosinophilia. Transfer of an expanded population of cells after CpG treatment, including IL-10-secreting follicular B cells, protected against eosinophilia.

CONCLUSIONS. CpG treatment provided B cell-mediated regulation of immune responses and B cell differentiation in CpG-induced immune remodeling with the use of IL-10. (*Invest Ophthalmol Vis Sci.* 2009;50:1626–1635) DOI:10.1167/iovs.08-2701

Allergic diseases are among the major health concerns in the Western world. The economic cost of managing allergic disease is high, ranking among the top four cost drains on the health care budget of the US government. Therefore, the development of new and effective therapies for allergic diseases is an immediate concern for health care providers.^{1,2} Because immunostimulatory CpG sequences were found to redirect or

suppress allergic immune responses in preclinical and early clinical trials, the study of their mechanism of action is of great interest.

Immunostimulatory CpG sequences were discovered after pioneering studies of Coley et al.³ that showed bacteria or their extracts conferred antitumor immunity in patients. Tokunaga et al.⁴ reported that the DNA and endotoxins of bacteria contributed to the immunostimulatory properties of bacteria. This led to the finding that unmethylated CpG sequences with the R1R2CGY1Y2 motif, with R1 being a purine-preferably G, R2 being a purine or T, and Y1 and Y2 being pyrimidines, confer immunostimulatory activity.⁵

CpG sequences have pleiotropic effects on many cell types, such as antigen-presenting cells, B cells, NK cells, and mast cells. CpG sequences are classified into three distinct groups: A class, B class, and C class.⁶ B-class oligonucleotides are potent adjuvants, skew the T-helper (Th) response to the Th1 type, and strongly activate B cells. B-class oligonucleotides also promote the maturation of plasmacytoid dendritic cells (pDCs); they have been used for anti-allergy vaccinations and are in clinical trials for allergic rhinitis. B-class nucleotides activate and induce the secretion of IL-12 and IL-18 by macrophages and DCs.⁷

The immunostimulatory sequence oligodeoxynucleotide (ISS-ODN) is a well-documented B-class oligonucleotide that has multiple immunostimulatory and immunoprotective effects. We have found that topical administration of ISS-ODN effectively inhibits the early-phase response (EPR) and the late-phase response (LPR) of ocular allergy.⁸ Although numerous studies have shown that CpG sequences have significant potential as anti-allergy medication, the mechanisms regarding how the CpG sequence exerts its potent allergy-suppressive effects have not been fully determined.

Part of the action of ISS-ODN involves signaling from the receptors of the CpG sequences, the Toll-like receptor 9 (TLR9).^{9,10} Its downstream adapter protein, MyD88, redirects the maturation of Th toward Th1 responses.⁷ MyD88 signals have been suggested to have a nonredundant role in driving regulatory activity in B cells.^{11,12} Mechanistically, CpG sequences activate macrophages to express elevated levels of IL-12, IL-18, IFN-α, and IFN-β.^{13,14} CpG sequences also stimulate NK cells to release IFN-γ and IL-12, which may help bias the T-lymphocytic responses.

In an earlier study, we reported that intraperitoneal or topical administration of CpG exerts potent anti-inflammatory activity in an established allergic conjunctivitis model.⁸ In addition to the direct effects on local immune cells, CpG sequences are known to induce hematopoietic remodeling^{15,16} with significant expansion of B-lineage cells. In contrast to their potent effects in preventing allergic responses, B cells are considered to be coordinators of eosinophilic inflammatory responses.

Based on these findings, we hypothesized that CpG exposure not only will stimulate immunocompetent cells but will lead to hematopoietic remodeling; both processes play a role in anti-inflammatory effects. To test this hypothesis, we searched for systemic changes that might contribute to the

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anti-inflammatory effects. We show here that CpG exposure led to changes in cytokine gene expression with a sustained induction of IL-10 in the inductive and effector tissues, together with a transient induction of IL-1ra, IL-18, and IL-12, depending on the tissue and kinetics. In addition, the secretion of IL-10 was exclusively mediated by CpG-induced follicular B cells.

MATERIALS AND METHODS

Animals

Eight-week-old female SWR/J mice and homozygous IL-10-deficient mice on a C57BL/6 or 129 background—age- and sex-matched to wild-type control mice—were obtained from the Jackson Laboratory (Bar Harbor, ME). The procedures used conformed to the principles for laboratory animal research outlined by the Animal Welfare Act, the National Institutes of Health guidelines for the experimental use of animals, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of Experimental Allergic Conjunctivitis

To induce experimental allergic conjunctivitis, mice were sensitized to short ragweed pollen by an established protocol.^{8,17} In brief, a 50- μ g suspension of ragweed pollen (ICN, Aurora, OH) and 1 mg aluminum hydroxide (Sigma, St. Louis, MO) was injected into the left hind footpad under general anesthesia. On day 21, conjunctivitis was induced by topical instillation of 1.5 mg ragweed suspended in 10 μ L phosphate-buffered saline (PBS) onto one eye. For each C57BL/6 mouse, a 50- μ g suspension of ragweed extract and aluminum hydroxide was injected intraperitoneally on day 0, week 2, and week 4, together with the initial footpad injection. The mice were challenged 6 weeks later.

CpG Oligonucleotides Administration

Endotoxin-free, B-class CpG oligonucleotide, a phosphorothioate ISS-ODN (Dynavax, Berkeley, CA) with sequences of 5'-TGACTGT-GAACGTTTCGAGATGA-3', and control oligonucleotide (5'-TGACTGT-GAAGGTTAGAGATGA-3') were prepared as described.¹⁸ To test the therapeutic efficacy of CpG on allergic conjunctivitis, CpG (100 μ g/mice) was injected intraperitoneally 3 days before the final ragweed challenge.

Evaluation of Late-Phase Responses

LPR intensity was quantified by counting the number of cells that infiltrated the conjunctiva.^{8,19} LPR is characterized strong infiltration of eosinophils into the conjunctiva; therefore, the number of eosinophils was taken to represent the intensity of the LPR.

Twenty-four hours after ragweed challenge, the mice were killed, and the eyes were enucleated with the attached lids and conjunctiva. Eyes and tissues were immediately fixed in 4% paraformaldehyde. The tissue was then embedded (Historesin; Leica Instruments GmbH, Heidelberg, Germany) and sectioned at 3 μ m thickness. Serial sagittal sections were stained with Giemsa or hematoxylin and eosin. The number of eosinophils in five consecutive conjunctival sections was determined in one 400 $\times$ field (4 fields/section) by a masked observer.

RNase Protection Assay

Conjunctiva, cervical lymph nodes, and spleen were obtained from four or five animals for each experimental group and were prepared for RNA extraction. Tissues were homogenized in a tissue grinder, and total RNA was extracted by homogenization (RNA Stat 60; Teltest Inc., Friendswood, TX). An RNase protection assay was performed using probes of mCK-2b panels (PharMingen, San Diego, CA) for the detection of IL-12p35, -12p40, -10, -1 α , -1 β , -1ra, -18, -6, IFN- γ , MIF, L32, and GAPDH.

CpG Oligonucleotide Treatment and Harvesting of Cells for Adoptive Transfer

To isolate splenocytes from CpG-treated mice, ragweed-sensitized mice were injected intraperitoneally with 100 μ g CpG oligonucleotide, and the spleen was aseptically collected 3 days after injection. Single-cell suspensions of the splenocytes were prepared after hypotonic lysis of the red blood cells.

For the adoptive transfer experiments, a suspension of splenocytes (10^6 cells/mouse) was injected into the mice through the tail vein. The viability of the isolated cells in the suspension was greater than 99%, as determined by the Trypan blue dye exclusion test.

Flow Cytometric Analysis

Splenocytes were labeled by incubation with monoclonal antibodies for 15 minutes at 4°C and were analyzed by fluorescence-activated cell sorting (FACSCalibur; Becton Dickinson, San Jose, CA). The monoclonal antibodies used are shown in Supplementary Table S1, <http://www.iovs.org/cgi/content/full/50/4/1626/DC1>. To block nonspecific binding, the cells were incubated in anti-Fc γ R/II/III antibody (Cedarlane, Horsham, PA) before antibody labeling.

Assay of IL-10 Secretion and Separation of IL-10-Secreting Cells

Mice were injected with 100 μ g CpG or a control oligonucleotide. Splenocytes were isolated 3 days after the injection and labeled with IL-10 catch reagent to assay for IL-10 secretion according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA). IL-10-secreting cells were additionally labeled by incubation with phycoerythrin (PE)-conjugated anti-IL-10 antibody and analyzed by fluorescence-activated cell sorting (FACSCalibur; Becton Dickinson). PE-conjugated anti-KLH antibody (eBioscience, San Diego, CA) was used as an isotype control.

In separate experiments, IL-10-secreting cells labeled with PE were magnetically isolated using anti-PE micro beads (Miltenyi Biotec). IL-10-secreting cells were stained for cell surface markers and analyzed by fluorescence-activated cell sorting (FACSCalibur; Becton Dickinson). Dead cells were excluded from analysis by gating out cells stained by propidium iodide.

For the adoptive transfer of IL-10-secreting B cells, splenocytes were first collected from CpG- or control oligonucleotide-injected sensitized mice. The B-cell fraction was separated by negative selection with a B-cell isolation kit (Miltenyi Biotec). The separated B-cell fraction was assayed for IL-10. IL-10-secreting or IL-10-nonsecreting B-cell fractions were separated with anti-PE microbeads.

Statistical Analysis

Data were analyzed with the Mann-Whitney *U* test or ANOVA, and differences were considered significant when *P* < 0.05.

RESULTS

Cellular Basis for Splenomegaly in CpG-Treated Animals

Initially, we determined the degree of splenocyte expansion in short ragweed-immunized mice before and after CpG oligonucleotide treatment. The kinetics and nature of the expansion of splenocytes were analyzed by fluorescence-activated cell sorting (Fig. 1). Immunized mice were challenged 2 weeks after sensitization, and spleens were collected. Mice were injected intraperitoneally with ISS-ODN or control oligonucleotide 1 day before challenge. Six days after treatment, consistent with previous reports, spleen weights in immunized mice were three times those in control mice (data not shown). Marked proliferation of hematopoietic progenitors and large immature blasts was observed after CpG treatment. The increase in total

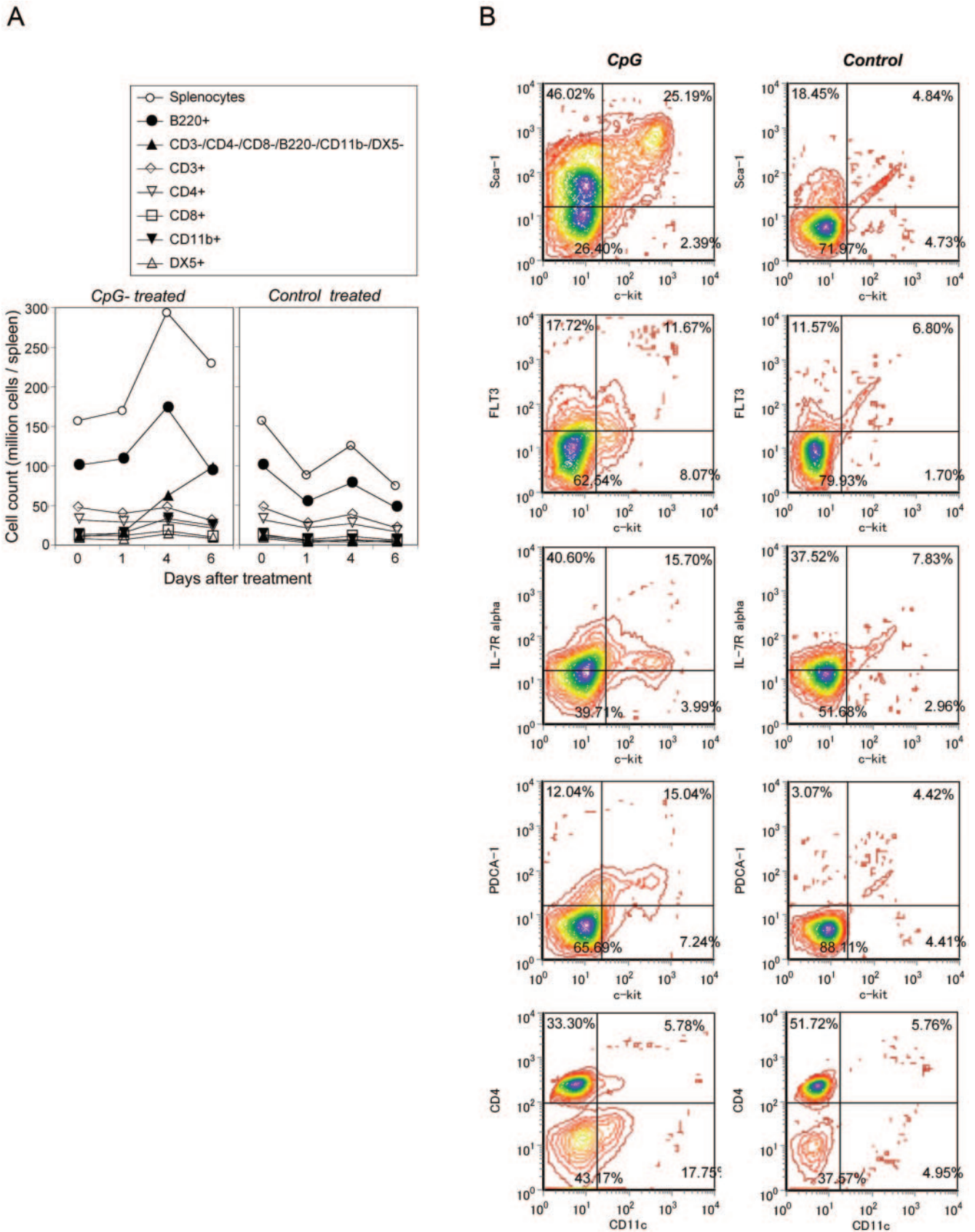


FIGURE 1. Expansion of splenocytes after intraperitoneal injection of CpG. Two weeks after ragweed sensitization, allergen-challenged mice were killed, and spleens were collected. CpG or control oligonucleotide was injected on the indicated day before allergen challenge. The kinetics of proliferation of the splenocyte subsets was analyzed by fluorescence-activated cell sorting. (A) B220⁺ cells showed a marked increase on day 4, whereas a lineage-negative subset (CD3⁻ CD4⁻ CD8⁻ B220⁻ CD11b⁻ DX5⁻) expanded significantly on day 6. *n* = 2 or 3 per time point. (B) Lineage analysis of CpG-stimulated splenocytes. The splenocytes were collected from CpG-treated-mice on day 4 and were subjected to fluorescence-activated cell sorting analysis. Sca-1⁺ c-kit⁺ subsets are notably expanded. PDCA-1⁺ cells are also expanded.

splenocytes peaked at 4 days at twice that of controls and then decreased (Fig. 1).

The CpG DNA receptor TLR9 is expressed on B cells, macrophages, and pDCs.²⁰ B220⁺ cells were the splenocytes with the highest level of expansion and accounted for 60% of the total increase on day 4. On day 6, the number of B220⁺ cells decreased to 30% of the total, but a decrease in the viability of these cells was not observed by trypan blue staining even 1 week after treatment. These observations suggested that stimulated B220⁺ cells migrated continuously from the spleen after stimulation.

A second major population of cells that increased was lineage-negative or immature cells (e.g., CD3⁺CD4⁺CD8⁺B220⁺CD11b⁺DX5⁺ cells). They constituted 65% of the total splenocytes increase on day 6. Together with the kinetics of the stimulated expansion of B220⁺ cells, their continuous increase in the spleen for 1 week suggested that immature cells may be retained in the spleen, probably until the acquisition of emigration properties after differentiation.

To further characterize the expansion of lineage-negative cells and lineage marker-expressing cells, the CpG-stimulated splenocytes were further tagged using hematopoietic differentiation markers. Fluorescence-activated cell sorting analyses of the entire population of cells 4 days after the injection of CpG are shown in Figure 1B. The most striking expansion was observed in Sca-1⁺ cells. The Sca-1⁺ population constituted 71.2% of the CpG-stimulated splenocytes, whereas most control splenocytes were Sca-1⁺.

To understand how CpG affects the differentiation of lymphoid and myeloid cells from Sca-1⁺ c-kit⁺ hematopoietic stem cells, the expression of stage-restricted markers was determined. The Sca-1⁺ c-kit⁺ cells constituted up to 46.02% of the expanded population (Fig. 1B). Because the Sca-1⁺ splenocytes were largely B220⁺, the expanded immature population induced by CpG exposure was considered to be progenitors of Sca-1⁺ B220⁺ B cells.

Previously, splenocytes treated with CpG were shown to confer granulocyte-macrophage colony forming activity or CTL function to sublethally irradiated mice.¹⁶ The Sca-1⁺ c-kit⁺ population (25.19%) of cells may contain expanded hematopoietic stem cells after stimulation by CpG. Hematopoietic stem cells can generate non-self-renewing hematopoietic multipotential progenitors, which are characterized as lineage-negative B220⁺ c-kit⁺ Sca-1⁺ Flt3⁺ cells.²¹

Consistent with the marked expansion of B-cell lineage cells, we also observed an expansion of IL-7R α ⁺ c-kit⁺ cells (15.70% after CpG treatment). These cells probably represented the common lymphoid progenitor or pro-B cell lineage. In contrast, the T-cell lineage populations were not affected in the total cell count. Rather, CD4⁺CD11c⁺ cells were proportionally decreased, consistent with previous evidence that T-cell lineage cells usually do not express TLR9.

Another subset of cells that express TLR9 is pDCs. In our analyses, we observed a massive expansion of the PDCA-1⁺ subset of cells together with the expansion of CD11c cells, which confirmed the expansion of pDCs. The subset of cells in this lineage also expressed c-kit at 15.04% for CpG-treated mice and 4.42% for controls. A sevenfold increase over control was also observed for CD11b⁺ cells. However, the limited cell numbers (approximately 10% of the splenocytes) compared with B220⁺ cells or lineage-negative populations suggested that their actual roles were limited. Taken together, these findings indicate that CpG treatment activates hematopoietic stem cells, leading to the differentiation of B cells and cells of myeloid lineage.

Adoptive Transfer of Protection against Late-Phase Inflammation

Our results suggested that CpG treatment can drastically alter systemic immunity. Results of earlier studies indicate that the induction of long-term protection by CpG sequences may promote the generation of a regulatory subset of cells in peripheral tissues and in the systemic arm. Initially, we hypothesized that regulatory cells are generated in the spleens of CpG-treated animals because of marked population changes. To test this hypothesis, we investigated whether splenocytes derived from CpG-treated mice conferred anti-inflammatory ability to sensitized mice. CpG-treated splenocytes from allergen-sensitized mice were adoptively transferred to allergen-sensitized mice. Recipients were then challenged with allergen exposure 3 days after transfer. The regulatory effects of the splenocytes on the late-phase allergic inflammation were evaluated 24 hours after allergen challenge. Initially, the recipients of CpG-treated splenocytes had slightly heavier spleens than did recipients of control oligonucleotide-treated splenocytes (spleen/body weights: CpG, 5.2 $\pm$ 0.3; control oligonucleotide-treated, 4.0 $\pm$ 0.2; $\times 1000$; $P = 0.01$), suggesting that the transferred splenocytes repopulated in the spleens of the recipients. After allergen challenge, the control-transferred mice showed significant conjunctival eosinophilia, whereas the conjunctival eosinophil number was significantly reduced in CpG-treated splenocyte recipient mice (Fig. 2). These findings indicated that there was an induction of regulatory cells after CpG treatment. Sensitized mice without adoptive transfer had similar allergic responses to control transferred mice.

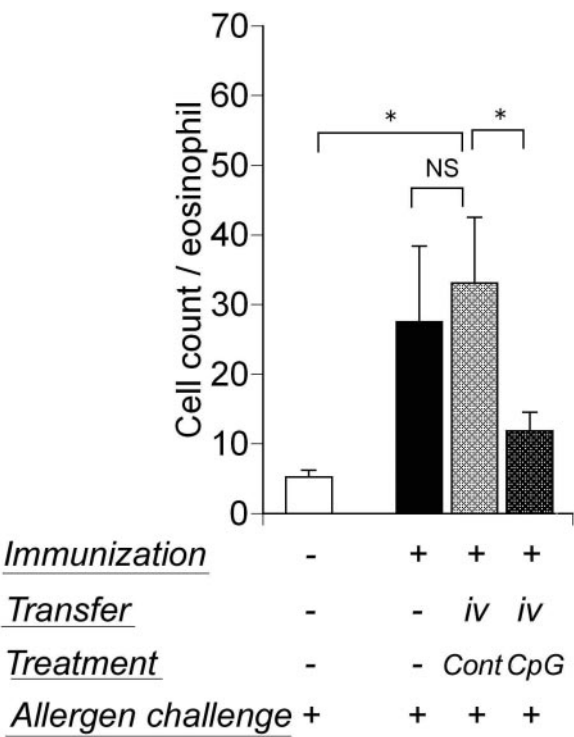


FIGURE 2. Suppression of allergen-induced eosinophilia by adoptive transfer of splenocytes from CpG-treated mice. Splenocytes were intravenously transferred from CpG- or control-treated mice, and the recipients were evaluated for eosinophil attraction 24 hours after challenge ($n = 9$ /group; $P < 0.05$). Transferred splenocytes from CpG-treated mice conferred protection against allergic responses.

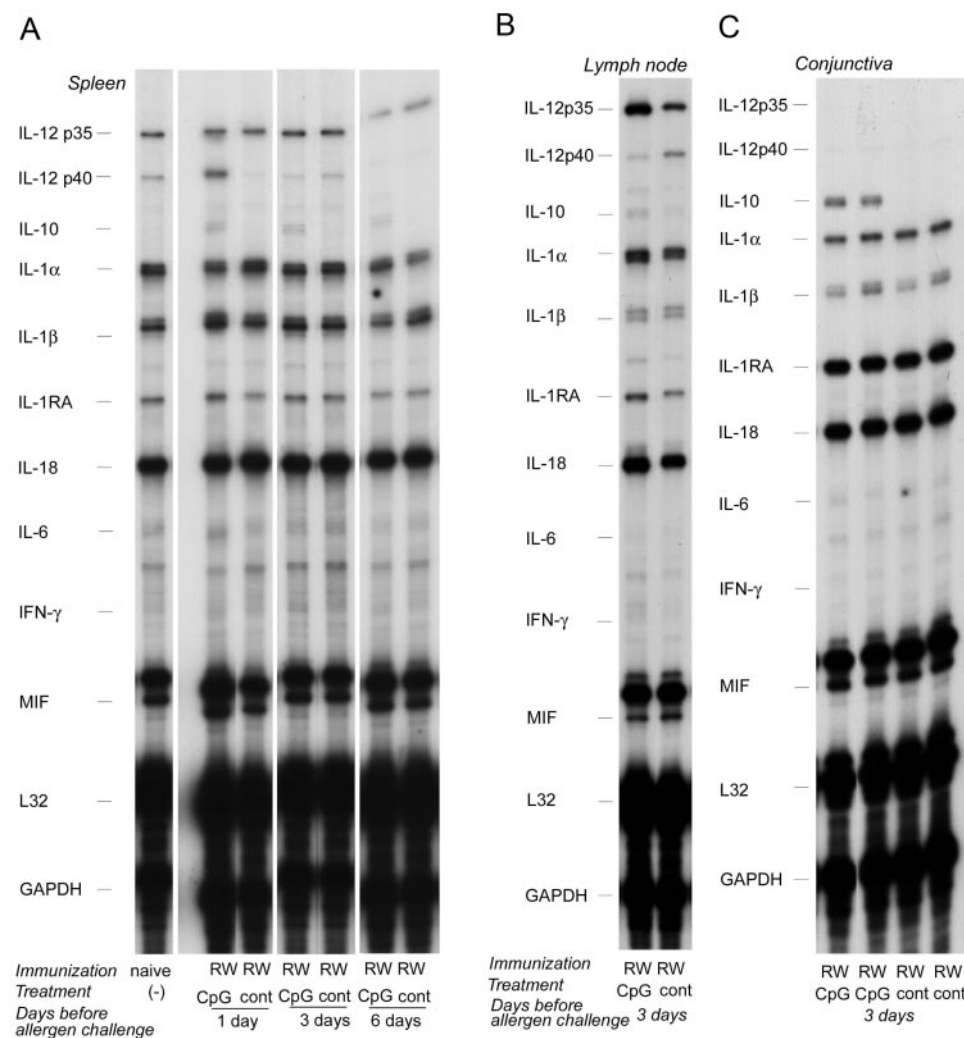


FIGURE 3. Induction of IL-10 in spleen (A), draining lymph nodes (B), and conjunctivas (C) of mice treated with CpG. To evaluate the kinetics of the responses to CpG, the CpG oligonucleotide was injected into sensitized mice 1, 3, and 6 days before the final allergen challenge. Extracted RNA from the spleen 24 hours after allergen challenge was assayed for RNase protection assay using mCK-2b probe (A, left). Sustained induction of IL-10 is observed for 6 days after CpG treatment and on 1 day after (A, right) draining lymph node (B) and conjunctiva (C). CpG was injected into sensitized mice 3 days before the final allergen challenge. Extracted RNA from the draining lymph node (B) and conjunctiva (C) 24 hours after allergen challenge was assayed for RNase protection assay using the mCK-2b probe.

Regulatory Cytokine Induction by CpG Treatment

The massive hematopoietic remodeling of the treated spleen and the transferability of regulatory function suggested that CpG may exert its immunoregulatory effect by altering the general nature/tendency of the immune system (immune deviation), especially in the spleen. To expand our initial findings and to test our hypothesis, we next analyzed cytokine expression profiles in CpG-treated animals after allergen challenge (Fig. 3).

IL-12 is a known contributor to the activity of the CpG sequences.⁷ For IL-12, the p35 subunit is synthesized by many cell types, whereas the p40 subunit is synthesized mainly by activated macrophages and DCs and appears restricted to cells that produce the biologically active heterodimer. Our data showed that cells derived from the spleens of CpG-treated mice were enhanced by an increase in the number of p40 cells on day 1 (Fig. 3A). However, the increase was lost thereafter, and an induction of p35 was not observed. Among the lymph node-derived cells, p40 was suppressed while p35 was increased (Fig. 3B). A slight increase of p40 was observed in the conjunctiva (Fig. 3C). Collectively, the induction of IL-12 appeared to be restricted to a certain time period in the spleen. These data strongly suggested that an alternative mechanism was activated.

We next searched for candidate molecules that are upregulated in the spleen after CpG treatment. For anti-inflammatory or immune-deviating cytokines, the inductions of IL-1ra, -18,

and -1 α were altered. Of these, IL-1ra was transiently induced in splenocytes (day 1; Fig. 3A) and lymph node cells (day 3; Fig. 3B). Transient induction of IL-18 and -1 α was observed among the lymph nodes cells but was not detected for splenocytes or conjunctival cells. These findings indicated that the induction of these mediators may not fully explain the prolonged effects of CpG.

Interestingly, the most prevalent and prolonged induction was observed for IL-10. Cells derived from the spleens and draining lymph nodes of CpG-treated mice showed enhanced IL-10 expression (Figs. 3A, 3B). Splenocytes from allergen-challenged mice did not show appreciable IL-10 induction (Fig. 3A). A marked induction of IL-10 was observed in the conjunctiva as an effector tissue (Fig. 3C). More importantly, the upregulation of IL-10 persisted for 1 week after CpG treatment in all these tissues, suggesting that CpG-induced IL-10 may play an indispensable role in CpG-mediated immune remodeling and in long-lasting therapeutic effects.

Requirements of IL-10 for CpG-Mediated Splenocyte Expansion

The modulation of systemic immunity by CpG treatment can be conveyed by expanded splenocytes (Fig. 2), indicating that the spleen is an important source of regulatory cells. In addition, IL-10 is consistently induced in the spleen. We observed that the most significantly expanded population of cells after CpG treatment was B220⁺ (Fig. 1). IL-10 is known to be an

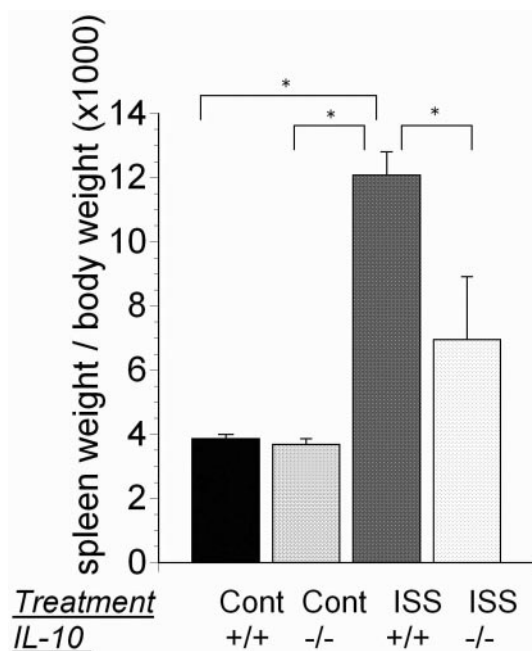


FIGURE 4. Requirement of IL-10 for CpG-induced splenomegaly. Splenomegaly induced by CpG treatment is suppressed in IL-10-deficient mice. Immunized mice challenged with short ragweed after CpG treatments were killed, and spleen weights were measured. $n = 9/\text{group}$. * $P < 0.05$.

important differentiation mediator for B-cell lineage cells. Therefore, we hypothesized that IL-10 may play a direct immune stimulatory role in splenocyte expansion. To test this hypothesis, IL-10-deficient mice were analyzed for splenomegaly 3 days after CpG injection. IL-10-deficient mice showed a significantly greater reduction of CpG-induced splenomegaly than wild-type mice (Fig. 4). In control-treated animals, IL-10 deficiency did not affect spleen size. We did not observe a reduction of splenocyte numbers in IL-10-deficient mice. Taken together, these results indicated that IL-10 contributes to the differentiation and expansion of allergen-primed cells in a CpG-dependent manner.

Exacerbation of Late-Phase Inflammation in Allergic Conjunctivitis by IL-10 Deficiency

We have reported that CpG treatment suppresses the clinical symptoms of immediate hypersensitivity reaction and

eosinophil recruitment in the late phase.⁸ To understand the roles of IL-10 in ISS-mediated immune regulation, we analyzed whether IL-10 also controls late-phase inflammatory cell recruitment in the allergic conjunctivitis model using IL-10-deficient mice. Sensitized wild-type mice showed allergen-dependent conjunctival eosinophilia (Fig. 5). Disruption of IL-10 exacerbated conjunctival eosinophilia significantly. Allergen-independent responses were not affected by IL-10 disruption. When IL-10-deficient mice were treated with CpG, the treatment failed to confer sufficient protection for IL-10-deficient mice, suggesting that IL-10 is also operative in limiting late-phase inflammatory responses after CpG treatment.

Characterization of IL-10-Secreting Cells in CpG-Treated Mice

IL-10 is an important candidate for therapeutic intervention for allergic and autoimmune diseases. Our data showed that IL-10 is required for CpG-mediated splenocyte expansion and is involved in limiting allergic inflammation. To further dissect the role of IL-10 in CpG-mediated immune remodeling/regulation, detailed analyses of IL-10-secreting cells, a potential candidate regulatory subset of cells, are required. We used an IL-10 secretion/separation assay and fluorescence-activated cell sorting analyses, which allowed the characterization of actively secreting IL-10 cells. In this assay, the level of secreted IL-10 was detected by the fluorescence intensity of the IL-10⁺ axis in scatterplots.

Initially, splenocytes collected 3 days after CpG treatment were analyzed with fluorescence-activated cell sorting. CpG treatment induced a marked expansion of higher scattering cells in the forward and side regions (R1; Fig. 6A). The IL-10-secreting populations, notably observed after CpG treatment, corresponded to the expanded R1 region. To determine the subset of cells that secreted IL-10 after CpG treatment, IL-10 secretion assay and fluorescence-activated cell sorting analysis of lineage marker expression were used. Fluorescence-activated cell sorting analysis showed that IL-10 was secreted primarily by B-220⁺ cells in the expanded population (Fig. 6B). In control oligonucleotide-treated spleens, a significantly lower level of IL-10 secretion was detected. Although IL-10 is known to be a canonical mediator of regulatory T cells, IL-10-secreting CD3⁺ cells did not significantly expand, and the secretion level was significantly lower than in B-cell lineage cells (data not shown).

CpG-induced IL-10-secreting cells were further typed as B-220⁺CD19⁺CD23⁺IgM⁺, indicating that they were follicular B cells (Fig. 6C). IL-10-secreting follicular B cells were primar-

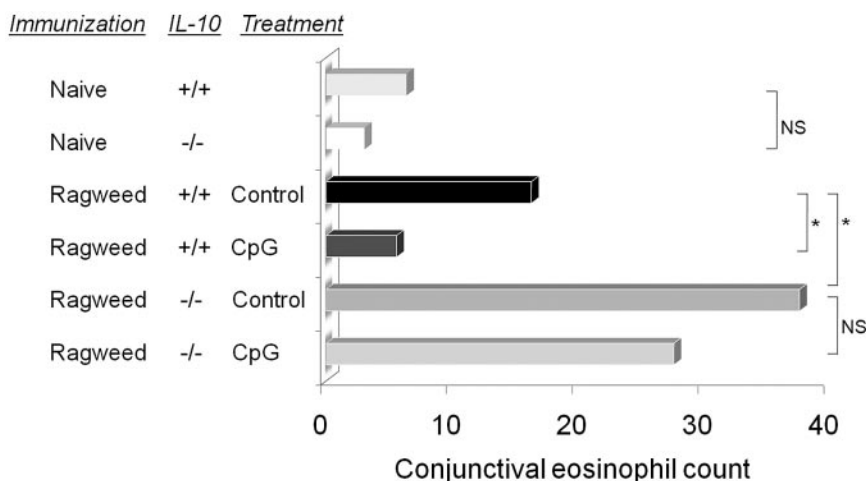


FIGURE 5. Exacerbated eosinophil recruitment in IL-10-deficient mice. Sensitized mice were analyzed for eosinophil attraction 24 hours after allergen challenge. IL-10 deficiency exacerbated allergen-dependent eosinophil attraction. CpG treatment did not confer protection for IL-10-deficient mice. $n = 10/\text{group}$. * $P < 0.05$.

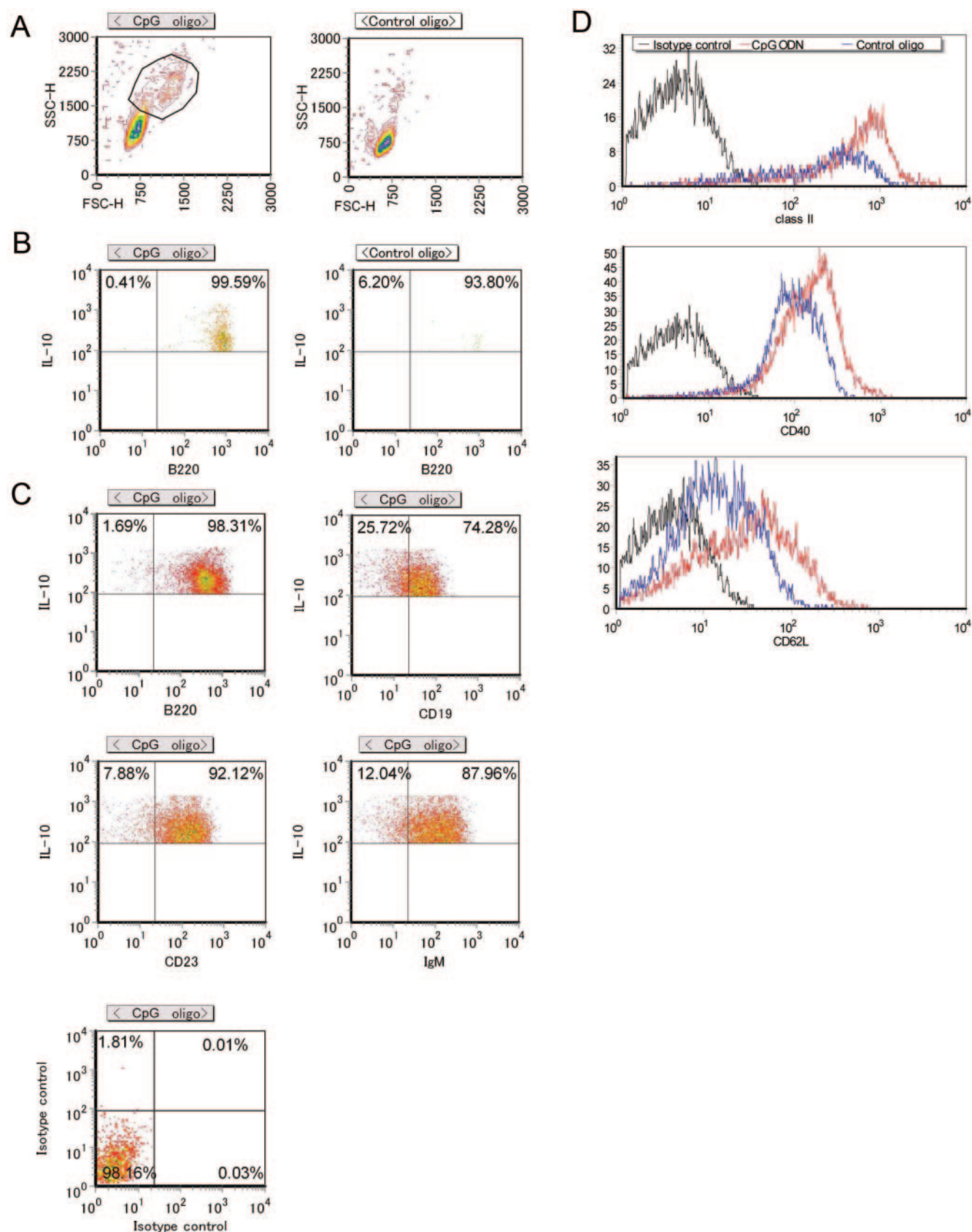


FIGURE 6. Characterization of IL-10-secreting cells induced by CpG treatment. (A) CpG treatment induced the expansion of splenocytes with higher forward- and side-scatter profiles in region 1 (circled), whereas the small lymphocyte region with low side scatter did not expand. (B) IL-10 secretion assay was performed and analyzed by fluorescence-activated cell sorting. Dead cells were excluded by propidium iodide staining. Expanded populations were analyzed for IL-10 secretion. CpG-induced IL-10-secreting populations were B220⁺ cells. (C) The IL-10-secreting cells

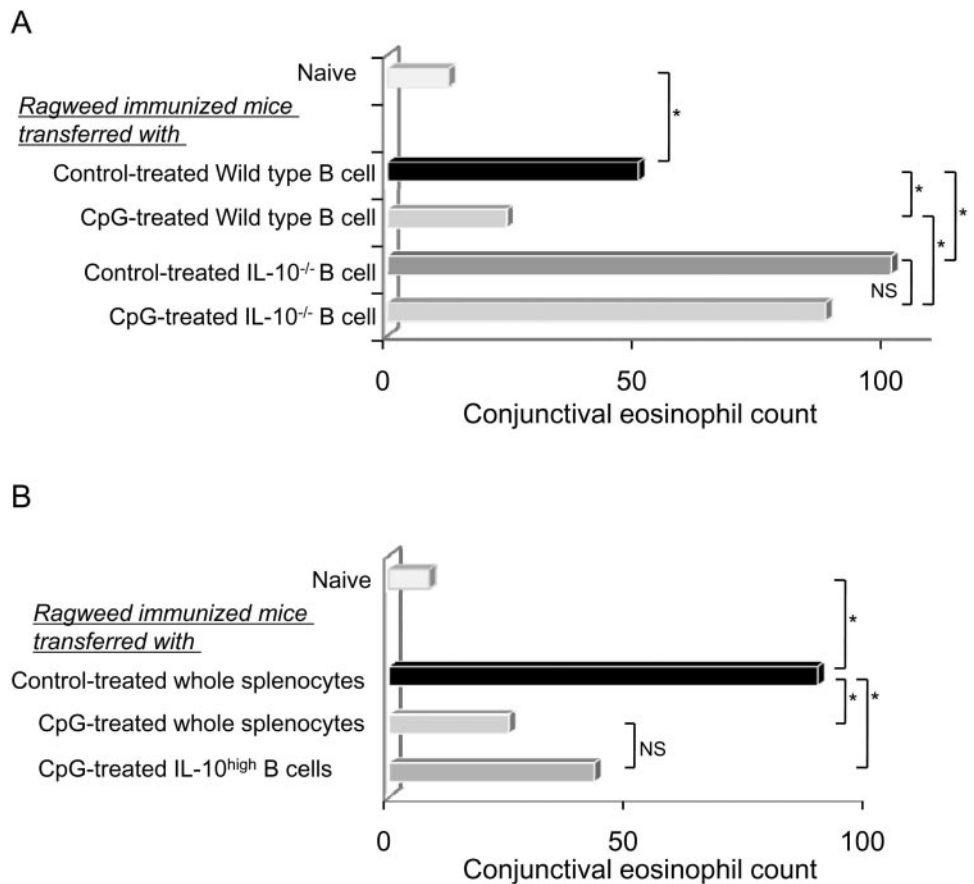


FIGURE 7. Protection and requirement of IL-10 for late-phase responses conferred by adoptive transfer of CpG-induced B cells. **(A)** Adoptive transfer of B cells from CpG-treated mice significantly suppressed conjunctival eosinophilia. B cells from allergen-sensitized IL-10-deficient mice exacerbated the eosinophilia. CpG treatment to donor IL-10-deficient mice did not confer appreciable protection to B cells. **(B)** Adoptive transfer of IL-10-secreting B cells (IL-10^{high} B cells) significantly suppressed conjunctival eosinophilia. $n = 10/\text{group}$. * $P < 0.05$; ** $P < 0.01$.

ily CD62L⁺, suggesting that they have a homing capability to peripheral lymphoid tissues (Fig. 6D). Because IL-10 production by B cells required a combined stimulation of B-cell receptor and CD40,^{22,23} we next evaluated the CD40 expression level. High expression levels of CD40 and major histocompatibility complex (MHC) class II, compared with levels in control-treated mice, showed that CD40 and MHC class II were strongly activated (Fig. 6D) and might have served as efficient antigen-presenting cells with presumable IL-10 secretion capability.

Roles of CpG-Generated Regulatory Subsets in LPR of Immediate Hypersensitivity

Next, we investigated the role played by IL-10 in allergen-sensitized B cells using adoptive transfer. Viable 1×10^7 splenic B cells were collected from immunized IL-10-deficient or wild-type mice, transferred into allergen-sensitized wild-type mice, and challenged with allergen. Regulatory roles for the late-phase inflammatory responses were assessed by conjunctival inflammatory cell recruitment 24 hours after challenge. The transfer of CpG-treated B cells significantly reduced eosinophilic inflammation (Fig. 7A), indicating that CpG-expanded B cells have a regulatory role.

Interestingly, when sensitized IL-10-deficient B cells were transferred to immunized recipients, the allergen-induced eosinophilic responses were markedly exacerbated compared with transfer in wild-type, B-cell transferred mice (Fig. 7A). When B

cells were collected from CpG-treated IL-10-deficient mice, CpG treatment did not give the B cells with suppressive effect on the eosinophilic inflammation, compared with recipients of control-treated-IL-10-deficient B cells. Thus, although IL-10 intrinsically limited B cell-related eosinophilic inflammation, the regulatory effects of CpG treatment also required IL-10.

To evaluate the exact contribution of CpG-activated subsets of cells in the context of IL-10 secretion, we next assessed the functional role of IL-10-secreting B cells expanded by CpG treatment by the adoptive transfer approach. The IL-10-secreting B-cell fraction (corresponding number derived from one CpG-treated donor for one recipient) were collected from CpG-treated immunized mice and compared with the whole splenocyte fraction transferred. Consistent with the data in Figure 2, adoptive transfer of the whole splenocyte population from CpG-treated mice significantly suppressed conjunctival eosinophilia (Fig. 7B). The IL-10-secreting B-cell fraction provided significant protection against eosinophilic inflammation, confirming its regulatory role. Altogether, CpG treatment induced B-cell-mediated immune remodeling with IL-10, leading to the generation of regulatory B cells.

DISCUSSION

Our results showed that CpG administration induced splenomegaly, as reported by others.¹⁶ Interestingly, our data indi-

were determined to be mainly CD19⁺CD23⁺IgM⁺, indicating follicular B cells. Separation using irrelevant antibody (PE-conjugated anti-KLH) is shown as negative control (*left panel bottom*). Analysis of their activation level is shown by the expression of class II, CD40, and CD62L. **(D)** CpG-induced-IL-10-secreting cells highly expressed class II, CD40, and CD62L compared with control-treated cells. Similar results were obtained by repeated experiments.

cated that the immunoregulatory effects of CpG are mediated by the expanded splenocytes. This involved a marked expansion of B-cell lineage cells, indicating that they have immunoregulatory roles rather than pathologic roles in the allergic responses.

The critical roles played by IL-10-secreting B cells have been shown by Anderton et al.^{12,24} With the use of an experimental allergic encephalitis model, they showed that IL-10-secreting B cells suppressed type 1 autoreactivity. The suppression of the Th2-type immune responses by regulatory B cells was also shown.²⁵ With the use of an intestinal inflammation model, mediated by the IL-4-dependent Th2 pathway, Mizoguchi et al.²⁵ showed that IL-10-producing CD1d^{high} regulatory B cells mediate disease suppression. This subset of cells was characterized as CD21^{int}CD62^{low}IgM^{int}CD23^{high}. In anterior chamber associated immune deviation (ACAID), marginal zone B cells have regulatory roles.²⁶ However, marginal zone B cells are mostly sessile; they appear to be distinct from regulatory B cells induced by CpG. Taken together, the regulatory B cells may have a more diverse role than had been thought.

We found that the CpG-induced B cells, including IL-10-secreting follicular B cells, had regulatory properties. IL-10 is required to limit eosinophilic inflammation; however, other factors were also operating (Fig. 3). Indeed, CpG-treated IL-10-deficient B cells appeared not to have completely lost their protective effect on transfer compared with CpG-treated wild-type B cells (Fig. 7A), possibly suggesting that the primary role of IL-10 is to contribute to the remodeling differentiation of hematopoietic cells, which presumably induce nonuniform regulatory lineage cells.

We do not argue that other subsets of cells may serve independently or in concert. Other suppressive factors may be coproduced in the respective subset of cells. For example, the IL-12 secreted by DCs is well known to be required for the regulatory effects of CpG treatment.²⁷ In our hands, PDCA-1⁺ cells were significantly expanded in the non-B-cell fraction of splenocytes after CpG treatment (Fig. 1). Based on the proposed roles of CpG-activated pDCs, we assume that PDCA-1⁺ pDCs may also have regulatory functions canonically by IL-12.

Interestingly, CpG treatment expanded the Sca-1⁺ c-kit⁺ and Sca-1⁺ c-kit⁻ cells. This expansion, most likely of hematopoietic stem cells, is consistent with the finding that CpG stimulation induces splenic colony-forming unit activity and extramedullary hematopoiesis.¹⁶ The most marked expansion was that of Sca-1⁺ B220⁺ B-cell progenitor cells. Thus, the CpG-induced differentiation profile was heavily deviated to the B-cell differentiation pathway. Recently, an expansion of B cells was shown to be required for T-cell tolerance in ACAID,²⁸ possibly explaining the observed expansion of B-cell lineage cells and the generation of regulatory B cells by CpG.

Results from IL-10-deficient mice showed that IL-10 limits eosinophilic inflammation and provides anti-inflammatory capacity in an allergen-dependent manner (Figs. 5, 7). Interestingly, the role of IL-10 as a regulatory mediator is controversial. Previously, IL-10 was shown to confer protection from mast cell degranulation in a mouse model of allergic conjunctivitis.²⁹ This strongly supported a regulatory role of IL-10 in allergic conjunctivitis and, together with our recent report, showed that eosinophilic inflammation is dependent on mast cell degranulation in murine allergic conjunctivitis.³⁰ However, we cannot exclude the possibility that IL-10 is a coexpressed marker of regulatory cells and that other comediators actually confer regulatory roles in concert. IL-10 itself may not solely be regulatory. Previously, Fukushima et al.³¹ showed that IL-10 deficiency exacerbated the secretion of inflammatory cytokines and reduced the eosinophilic inflammation in the multiple allergen-exposed allergic conjunctivitis model. We assumed that this discrepancy arose from the pleiotropic nature

of IL-10.³² The multiple allergen exposure used by Fukushima et al.³¹ was expected to induce or activate the homing of hematopoietic lineage cells from the bone marrow, which could certainly be affected by IL-10 deficiency. IL-10 can play stimulatory roles by promoting B-cell differentiation or by class switching.³³ Additionally, IL-10 is known to be produced by the Th1 subset.³⁴ When inflammation is aggravated, Th1-type responses may also be involved.

In the arm of the indirect effect of CpG-induced IL-10, IL-10-secreting regulatory T cells have been reported to be indirectly generated by CpG treatment. Bilsborough et al.³⁵ showed that CpG-stimulated pDC-derived antigen-presenting cells can differentiate into IL-10-producing regulatory T cells from naive T cells. Alternatively, CD11c^{low}CD45RB^{high} DCs differentiated by IL-10 induce the differentiation of IL-10-producing regulatory T cells.³⁶ In addition, CpG and IL-10 may act indirectly by suppressing the induction of multiple cytokines by inducing SOCS1 or SOCS3.³⁷⁻³⁹

Our findings on the CpG-stimulated generation of regulatory B cells also implicate the pathologic aspects of immune responses, presumably by infectious agents. Parasites or viruses can elicit IL-10 production from B cells by TLR-dependent mechanisms. The induction of IL-10 production by B cells is used to dampen or subvert host defense mechanisms.¹² Thus, an understanding of B cell-mediated regulatory events might be beneficial in developing a more efficacious strategy against infectious diseases.

Taken together, we propose that a key mechanism of CpG action involves IL-10-mediated immune remodeling and the generation of regulatory B cells as well as other regulatory cells, systemically or locally. Our findings implicate clinically important issues about how and where CpG vaccinations must be performed for effective anti-allergy treatment.

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Table 1

FITC-anti-CD3e	BD Pharmingen
FITC-anti-CD4	BD Pharmingen
PerCP-anti-CD4	eBioscience
PerCP-anti-CD8	BD Pharmingen
FITC-anti-B220	BD Pharmingen
PerCP-anti-B220	BD Pharmingen
FITC-anti-CD11b	BD Pharmingen
FITC-anti-CD25	eBioscience
FITC-anti-CD19	eBioscience
FITC-anti-IgM	eBioscience
FITC-anti-IgD	eBioscience
FITC-anti-CD62L	eBioscience
FITC-anti-class II	eBioscience
FITC-anti-CD23	eBioscience
FITC-Sca-1	Biolegend
FITC-Flt-3	Chemicon
FITC-PDCA-1	Miltenyi Biotec
FITC-IL-7 receptor alpha	Biolegend
FITC-CD40	Biolegend
biotinized anti-CTLA-4	eBioscience
biotinized anti-DX5	eBioscience
FITC- or PerCP-conjugated IgG	BD Pharmingen
streptavidin-FITC	eBioscience
streptavidin-PE	eBioscience
streptavidin-PerCP	BD Pharmingen