

Cyclophosphamide augments inflammation by reducing immunosuppression in a mouse model of allergic airway disease

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Background: Allergic asthma is a T_H2 cell-driven immunological disease, characterized by eosinophilic inflammation. The cytotoxic agent cyclophosphamide paradoxically augments several immune responses.

Objective: We studied the proposal that cyclophosphamide may aggravate airway inflammation in allergic mice, and these features might result from the loss of regulatory T cells.

Methods: BALB/c mice were immunized with ovalbumin on days 0 and 14 and challenged with aerosolized ovalbumin from days 21 to 27. Some mice also received cyclophosphamide on days -2 and 12.

Results: In the lungs of cyclophosphamide-treated animals, pronounced worsening of inflammatory features was noted, including increased eosinophil infiltration, epithelial thickness, mucus occlusion, and eosinophil numbers in bronchoalveolar lavage fluid. There was also increased total and ovalbumin-specific serum IgE, increased IL-4 and IL-5 secretion by peritracheal lymph node cells, and reduced lung mRNA expression of IL-10 and TGF- β in animals treated with cyclophosphamide. The expression of FoxP3, a marker of regulatory T cells, was significantly reduced in lymphoid organs after the second injection of cyclophosphamide, and in the lung tissue after allergen challenge in cyclophosphamide-treated mice. Lung IL-10⁺CD4⁺ T cells and cytotoxic T lymphocyte-associated antigen 4⁺CD4⁺ T cells were reduced after allergen challenge in cyclophosphamide-treated mice. **Conclusion:** Cyclophosphamide worsened features of allergic pulmonary inflammation in this model, in association with increased production of IgE and T_H2 cytokines. The reduced expression of FoxP3 and immunosuppressive cytokines by cyclophosphamide is consistent with the possibility that toxicity to regulatory T cells may contribute to the increased inflammation. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Asthma, cyclophosphamide, eosinophils, IgE, IL-5, regulatory T cells, cytotoxic T lymphocyte-associated antigen 4, FoxP3, IL-10, TGF- β

The incidence of allergic asthma has considerably increased in recent decades, and since 1980 has nearly doubled in industrialized countries.¹ Airway hyperreactivity and chronic mucosal inflammation, mediated by CD4⁺ T_H2 cells, are characteristic features of this disease.² Other events associated with allergic asthma are mucus hypersecretion and airway remodeling.³ Complex interactions between inflammatory cells lead to the production of cytokines and other inflammatory mediators,⁴ including the T_H2 cytokines IL-4 and IL-5. Studies in murine models have demonstrated that IL-4 is essential for the production of IgE and T_H2 cytokines, and IL-5 is required for pulmonary eosinophilia.^{5,6}

Cyclophosphamide is a cytotoxic alkylating agent widely used for the treatment of neoplastic and severe autoimmune diseases. Paradoxically, cyclophosphamide has been reported to augment a wide variety of immune responses. Administration of cyclophosphamide potentiates delayed-type hypersensitivity⁷ and tumor rejection.⁸ In nonobese diabetic mice, cyclophosphamide administration promotes the susceptibility of mice to insulin-dependent diabetes mellitus (IDDM), and transfer experiments indicate that cyclophosphamide inhibits a suppressor response that normally prevents activation of effector T cells.⁹ The exacerbation of inflammatory responses and blockade of suppressive activity after cyclophosphamide treatment have suggested that cyclophosphamide preferentially depletes suppressor or regulatory T cells.^{9,10}

CD4⁺CD25⁺ regulatory T cells (Tregs), a distinct subset of CD4⁺ T cells,¹¹ are postulated to maintain peripheral self-tolerance. Depletion of Treg results in the development of organ-specific autoimmune diseases.¹² Many studies on Tregs have shown them to control T_H1-mediated diseases. However, there is recent evidence that they may also control T_H2-mediated diseases, such as allergic asthma.¹³⁻¹⁷ Naturally occurring Tregs specifically express the transcription factor FoxP3.¹⁸ Recently, other types of regulatory T cells induced after antigen stimulation have been described, including IL-10-secreting T-regulatory 1 cells¹⁹ and TGF- β -secreting T_H3 cells.^{20,21} Furthermore, immunosuppression mediated by

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Abbreviations used

Alum:	Aluminum hydroxide
BAL:	Bronchoalveolar lavage
CTLA-4:	Cytotoxic T lymphocyte-associated antigen 4
CY/OVA:	Cyclophosphamide/ovalbumin
FITC:	Fluorescein isothiocyanate
IDDM:	Insulin-dependent diabetes mellitus
PerCP:	Peridinin chlorophyll protein
PTLN:	Peritracheal lymph node
Treg:	Regulatory T cell

Treg includes the ligation of CD80 and CD86 on T cells by the cell surface molecule, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4).²²

Most of the immune responses potentiated by cyclophosphamide, such as IDDM, are associated with T_H1 cells. However, cyclophosphamide augments blood eosinophilia after antigen administration by increased production of the T_H2 cytokine IL-5.²³ A recent paper suggests that cyclophosphamide decreases the number and inhibits the function of naturally occurring CD4⁺CD25⁺ Treg.²⁴ We have investigated the effect of cyclophosphamide on a murine model of allergic pulmonary inflammation that exhibits a T_H2 response and eosinophilia. We report that cyclophosphamide augments T_H2 responses and airway eosinophilic inflammation and reduces expression of FoxP3, IL-10, and TGF- β in the lung.

METHODS

Animals

Female BALB/c mice, 6–8 weeks old, were obtained from Animal Resources Centre (Perth, Australia). All animals were housed in pathogen-free conditions at the Garvan Institute of Medical Research. All experiments were approved by the Garvan/St Vincent's Animal Experimentation Ethics Committee and conducted according to the animal welfare guidelines of the National Health and Medical Research Control of Australia.

Induction of asthma and administration of cyclophosphamide

Mice were immunized and boosted by intraperitoneal injection of 100 μ g ovalbumin (grade V; Sigma, St Louis, Mo) adsorbed onto 0.1 mL Al(OH)₃ gel (alum; Sigma) at day 0 and day 14 according to a mouse asthma model described previously.²⁵ Unimmunized control mice were injected with the same quantity of alum on day 0 and day 14, without ovalbumin. Cyclophosphamide-treated animals received 2 doses of 150 mg/kg cyclophosphamide (Sigma) intraperitoneally at day –2 and day 12. A moderate dose of cyclophosphamide is used in this study, sufficient to induce eosinophilia in a murine model.²³ All groups were challenged with aerosolized ovalbumin (1% wt/vol in PBS) on days 21, 23, 25, and 27 for 20 minutes each day. Mice were euthanized at day 28, and tissues and blood were taken for *in vitro* studies.

A time-course experiment was also included in which mice were given cyclophosphamide and ovalbumin as described and challenged with ovalbumin aerosol on days 19, 21, 23, and 25. Mice were euthanized at days 5, 12, 19, and 26 for *in vitro* studies.

Bronchoalveolar lavage and cytospin

Twenty-four hours after the last ovalbumin aerosol, mice were killed by carbon dioxide. The left lungs were ligated at the left main branch of the trachea by using surgical thread, and the right lungs were lavaged twice with 0.5 mL normal saline. Bronchoalveolar lavage (BAL) fluid was centrifuged at 1200 rpm for 5 minutes at room temperature, and cells were subjected to cytocentrifugation. Cytospin slides were fixed in methanol and stained with modified Giemsa. Cell differential percentages were determined by light microscopy. Total BAL cells were counted by using a hemocytometer, and absolute numbers of the cell types were determined.

Histological analysis

The lobes of the nonlabeled lung were used for histology and preparation of cell suspensions. One lobe was fixed in 10% formalin (Sigma) and embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff. All slides were read by an observer who was blind to the treatment groups, and severity of inflammation was quantitated on a 0–3 scale. The appearances in untreated mice were defined as 0, and maximum changes were scored 3. For eosinophil infiltration, a score of 0 = no eosinophils, 1 = occasional eosinophils, 2 = moderate numbers of eosinophils including some eosinophils adjacent to each other, and 3 = large areas of predominantly eosinophils. For epithelial thickness, 0 = thickness in controls, 1 = 1–1.5 $\times$ thickness in controls, 2 = 1.5–2 $\times$ thickness in controls, and 3 = >2 $\times$ thickness in control mice. For airway mucus occlusion, 0 = no occlusion, 1 = small areas of luminal mucus, 2 = partial occlusion of at least 1 small airway, and 3 = complete occlusion of at least small airway. To enumerate peribronchial eosinophils, in each section under low power, the 5 largest airways were identified, and the most and least cellular regions adjacent to each were selected. In each of these 10 regions, an area extending along the epithelium for 250 μ m and into the lung parenchyma for 50 μ m was identified. The number of eosinophils in each area was counted, and the mean of the 10 areas was determined.

Cell isolation and *in vitro* stimulation

Cell suspensions were prepared from peritracheal lymph nodes (PTLNs) and spleens. Cells were filtered through nylon mesh (Cell Strainer; BD Biosciences, Bedford, Mass). Cells were cultured at 10⁶ cells/mL in RPMI medium (Gibco Invitrogen, Grand Island, NY) supplemented with 10% (vol/vol) FCS (Hyclone, Logan, Utah), 1% L-glutamine (Gibco Invitrogen), 2% HEPES (Gibco Invitrogen), and 10 U/mL and 10 μ g/mL penicillin/streptomycin (Gibco Invitrogen). Cells were cultured for 48 hours at 37°C in a moisturized incubator with 5% CO₂ in air, in conditions of no stimulus or 10 μ g/mL ovalbumin.

ELISA

Total serum IgE was detected by using the Mouse IgE ELISA Set (BD Biosciences Pharmingen, San Diego, Calif) according to the manufacturer's instructions. Absolute concentrations were determined with the IgE manufacturer's standard. The lower detection limit was 5 ng/mL. Ovalbumin-specific IgE was detected by coating 384-well ELISA plates with 20 μ L of 20 μ g/mL ovalbumin overnight at 4°C, and then incubating with diluted sera. All subsequent steps were as for total IgE. Sera were diluted with PBS, 1:50 for total and 1:5 for ovalbumin-specific IgE. Cytokine concentrations in BAL fluids and culture supernatants were measured by using mouse IL-4 and IL-5 ELISA sets (BD Biosciences Pharmingen) according to the manufacturer's instructions, with lower detection limits of 7.8 and 15.6 pg/mL, respectively. BAL fluids used for cytokine detection were taken from the first lavage.

Flow cytometry

Two lobes of the nonlabeled lung from each animal were mechanically minced and digested with collagenase type I (0.5 mg/mL, C-0130; Sigma) in RPMI-1640 for 15 minutes at 37°C with vigorous pipeting. Aliquots of 10^5 cells were washed twice in 1% BSA/0.02% sodium azide. Cell surface staining was performed by incubation with peridinin chlorophyll protein (PerCP)-conjugated antimouse CD4 (CD4⁺-PerCP; clone L3T4), fluorescein isothiocyanate (FITC)-conjugated antimouse CD25 (CD25⁺-FITC), and phycoerythrin-conjugated antimouse CTLA-4 (CTLA-4⁺-phycoerythrin) mAbs (BD Biosciences Pharmingen) for 25 minutes on ice. Antibodies were titrated for optimal staining.

For intracellular staining of IL-10, 10^6 cells from lungs were stimulated for 6 hours with 5 µg/mL concanavalin A (Sigma). Two hours before harvest, Golgistop (BD Biosciences Pharmingen) was added according to the manufacturer's instructions. Cells were stained with antimouse CD4 (CD4⁺-PerCP) and antimouse CD25 (CD25⁺-phycoerythrin) mAb as described, permeabilized by using Cytofix/Cytoperm (BD Biosciences Pharmingen) according to the manufacturer's instructions, and stained with antimouse IL-10 (IL-10⁺-FITC) mAb for 30 minutes on ice.

Cells were centrifuged at 1200 rpm for 5 minutes at 4°C and resuspended in 1% BSA/0.02% sodium azide at 4°C before analysis on a FACScan flow cytometer using Cellquest software (Becton Dickinson, San Jose, Calif). The lymphocyte-gated region was determined on forward-scatter and side-scatter signals on control splenocytes. Cursors were positioned in cells stained with isotype control antirat IgG₁-FITC and IgG_{2a}-phycoerythrin mAb (BD Biosciences Pharmingen), with negatively stained cells >98%. Ten thousand cells in the lymphocyte gate were analyzed for each sample. CD4⁺ cells were gated, and the percentage of CD25⁺, CTLA-4⁺, and intracellular IL-10⁺ cells was determined.

CD4⁺ T-cell purification

Cell suspensions were prepared from spleens, thymuses, and PTLNs as described. Lung cell suspensions were obtained with collagenase as described. Cells were incubated with antimouse CD4 (L3T4) microbeads (Miltenyi Biotec Inc, Auburn, Calif) and separated with magnetic cell sorting (MACS) columns (Miltenyi Biotec Inc) according to the manufacturer's instructions. The resulting populations were checked by flow cytometry and were always greater than 95% CD4⁺.

Real-time PCR

RNA was extracted from purified CD4⁺ T cells from lungs, thymuses, spleens, and PTLNs by TRIzol reagent (Invitrogen Life Technologies, Carlsbad, Calif). RNA samples were purified by using RNase-free DNaseI (Qiagen, Clifton Hill, Australia) according to the manufacturer's instructions, and passed through RNeasy mini kits (Qiagen).

RNA (2 µg) was treated with avian myelomatosis virus reverse transcriptase (Promega, Madison, Wis) at 42°C for 1 hour by using oligo(dT)₁₅ (Promega). The cDNA was amplified for β-actin, IL-4, IL-5, ²³ IL-10, TGF-β, ²⁶ and FoxP3¹⁸ by using the quantitative TaqMan technique. Primer sequences are shown in Table E1 in this article's Online Repository at www.jacionline.org. cDNA was amplified with Platinum qPCR supermix-uracil-DNA glycosylase (Invitrogen Life Technologies), primers, TaqMan probe, and distilled water, according to the manufacturer's instructions. Real-time PCR was performed by using the Rotor-Gene 3000 four-channel multiplexing system (Corbett Research, Sydney, Australia).

PCR conditions were 3 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The threshold cycle was recorded as the cycle at which the reporter fluorescent emission

increased above a defined threshold. Each PCR product was confirmed by melting curve analysis and agarose gel electrophoresis. The amount of mRNA was measured as fold change relative to the amount in control untreated mice. To determine fold change, each sample was normalized against β-actin by obtaining the difference in threshold cycle number between β-actin and cytokine or FoxP3. This number was then subtracted from the mean normalized value of control untreated mice to generate the uncorrected difference. The fold change of each sample was then determined by raising the amplification efficiency (generated by the PCR machine) to the power of the uncorrected difference.

Statistical analysis

Data are given as means ± SEMs. The 2-tailed unpaired *t* test analysis was used to determine statistical significance, and the ANOVA repeated measures were used in analyzing flow cytometry results (Statview software 5.0; Abacus Concepts, Berkeley, Calif). *P* values less than .05 were considered statistically significant.

RESULTS

Cyclophosphamide exacerbated lung inflammation

Mice were immunized twice with ovalbumin and challenged 4 times by inhalation of aerosolized ovalbumin. Lung histology was assessed 24 hours after the last challenge. In these mice (ovalbumin group), high-power examination revealed significant cellular infiltration including numerous eosinophils in the perivascular and peribronchial areas (Fig 1, A). Sections of ovalbumin-immunized and challenged mice given 2 injections of cyclophosphamide (CY/OVA group) showed a much more pronounced cellular infiltrate in the perivascular and peribronchial areas containing very abundant eosinophils (Fig 1, B). In nonimmunized mice injected with alum intraperitoneally (alum group), there was minimal cellular infiltrate, and eosinophils were very rare (Fig 1, C). Unimmunized mice given 2 injections of cyclophosphamide (cyclophosphamide group) showed very mild inflammation with occasional eosinophils (Fig 1, D).

Semiquantitative scoring of sections revealed that eosinophils around bronchi and blood vessels, airway epithelial thickness, and airway mucus occlusion were significantly greater in CY/OVA mice than ovalbumin mice. In alum and cyclophosphamide mice, the scores were very low (Fig 1, E). Quantitation of peribronchial eosinophils revealed that CY/OVA mice had more than 3 times as many eosinophils as ovalbumin mice (34 ± 4.7 vs 11 ± 2.6 eosinophils per 250 µm of epithelium, respectively; mean ± SEM; *n* = 6; *P* = .0016). In unimmunized mice, eosinophil numbers were very low (0.08 ± 0.08 , 0.5 ± 0.2 , and 0 in alum, cyclophosphamide, and untreated groups, respectively).

Under low power, there was moderate cellular infiltrate in the ovalbumin group (see this article's Fig E1, A, in the Online Repository at www.jacionline.org), extensive infiltrate in the CY/OVA group (see this article's Fig E1, B, in the Online Repository at www.jacionline.org), and very little infiltrate in the alum or cyclophosphamide groups (see this article's Fig E1, C and D, in the Online

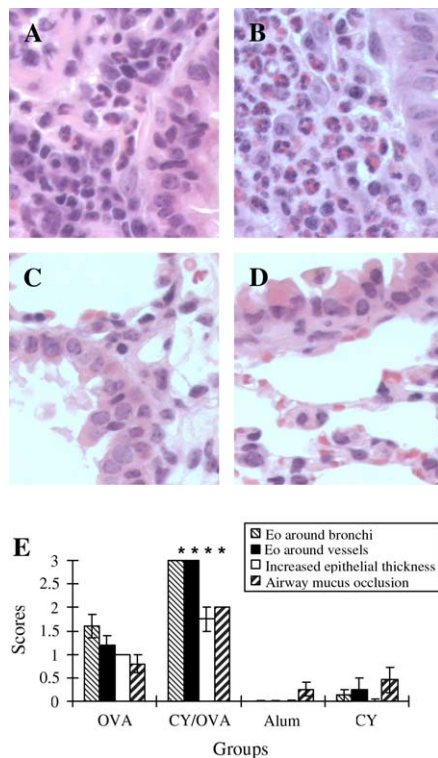


FIG 1. Lung histology. The H&E-stained sections are representative of lungs from (A) ovalbumin (OVA), (B) CY/OVA, (C) alum, and (D) cyclophosphamide (CY) mice at 400 $\times$ magnification. E, Semi-quantitation of histology. There were 6 mice in each group. Data are shown as means $\pm$ SEMs. In columns without error bars, SEM = 0. * P < .05 compared with OVA mice. The experiment shown is representative of 3 separate experiments. Eo, Eosinophils.

Repository at www.jacionline.org). There was essentially no infiltrate in completely untreated mice (see this article's Fig E1, E, in the Online Repository at www.jacionline.org), and under high power, no eosinophils could be detected (see this article's Fig E1, F, in the Online Repository at www.jacionline.org). In periodic acid-Schiff-stained sections, the ovalbumin group revealed evidence of mucus production (see this article's Fig E1, G, in the Online Repository at www.jacionline.org). Strikingly, the CY/OVA group showed a very remarkable amount of mucus that partially plugged all of the airway lumina (see this article's Fig E1, H, in the Online Repository at www.jacionline.org). The unimmunized alum and cyclophosphamide groups demonstrated little mucus (see this article's Fig E1, I and J, in the Online Repository at www.jacionline.org).

Cyclophosphamide increased cells in BAL fluid

CY/OVA mice had markedly increased total cell numbers in BAL fluid compared with ovalbumin mice. The numbers of eosinophils, macrophages, and lymphocytes were also much higher (Fig 2, A). The cell numbers in the alum and cyclophosphamide groups were lower than in the ovalbumin-treated mice. In both ovalbumin and CY/

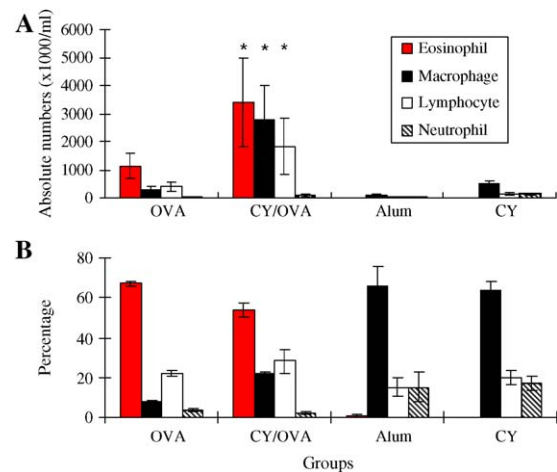


FIG 2. Cell types present in BAL fluid. Results are expressed as (A) absolute numbers and (B) percentages. There were 6 mice in each group. Data are shown as means $\pm$ SEMs. * P < .05 compared with ovalbumin (OVA) mice. The experiment shown is representative of 3 separate experiments. CY, Cyclophosphamide.

OVA mice, the percentage of eosinophils was very high, whereas very few eosinophils were observed in the 2 control groups (Fig 2, B).

Cyclophosphamide increased serum IgE and IL-4 and IL-5 production

Total serum IgE in the alum group was very low, but it was higher in the ovalbumin mice. In the CY/OVA mice, both serum total and ovalbumin-specific IgE were markedly increased. In unimmunized mice, cyclophosphamide did not increase IgE levels (Fig 3, A).

After incubation with ovalbumin, IL-4 and IL-5 secretion by PTLN cells was elevated in cultures from ovalbumin mice. Secretion of these cytokines was markedly increased in cultures from CY/OVA mice. In cultures from the alum and cyclophosphamide groups, little or no production of these cytokines was detected (Fig 3, B).

Cyclophosphamide decreased IL-10 and TGF- β expression in lung

In lung cDNA samples, IL-10 mRNA expression was increased in ovalbumin-immunized mice but was significantly reduced in CY/OVA mice. IL-10 did not increase in either alum or cyclophosphamide mice compared with control untreated mice (Fig 4, A). Similar findings were made for TGF- β expression, which was increased in ovalbumin mice, but was significantly lower in CY/OVA mice. In unimmunized cyclophosphamide mice, TGF- β production increased slightly compared with alum mice (Fig 4, B).

Effect of cyclophosphamide on FoxP3 expression

Mice were immunized on days 0 and 14, and some mice were given cyclophosphamide on days -2 and 12. Aerosol challenge was performed on days 19, 21, 23, and 25.

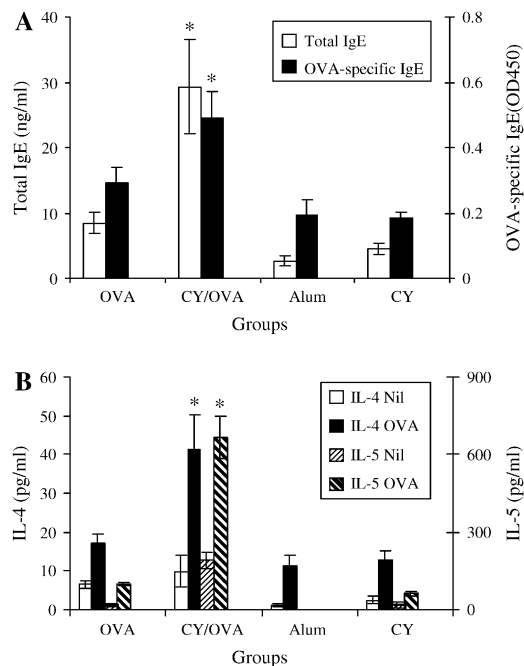


FIG 3. A, Total and OVA-specific serum IgE. B, IL-4 and IL-5 secretion by PTLN cells. Cells were cultured with no stimulus (Nil) or 10 μ g/mL ovalbumin (OVA) for 48 hours. Data are shown as means $\pm$ SEMs, 6 mice per group. * P < .05 compared with OVA mice. The data shown are representative of 3 separate experiments. CY, Cyclophosphamide.

FoxP3 expression in CD4⁺ T cells in spleen, thymus, and PTLN was significantly higher in CY/OVA mice at day 5 and significantly lower at day 19 (before challenge) compared with ovalbumin mice. FoxP3 expression in lymphoid organs at day 12 and 26 was not significantly different between CY/OVA and ovalbumin mice (Fig 5, A-C). In CD4⁺ T cells purified from the lung, FoxP3 expression was similar in ovalbumin and CY/OVA mice at day 19, before ovalbumin challenge, but significantly reduced in CY/OVA mice at day 26, after challenge (Fig 5, D).

Cyclophosphamide affected the proportion of CD4⁺CTLA-4⁺ cells and IL-10-producing CD4⁺ T cells in the lung

At day 26, total CD4⁺ T-cell numbers were significantly lower in CY/OVA mice compared with ovalbumin mice (Table I, A). The data suggest that cyclophosphamide is toxic to CD4⁺ lymphocytes. CD4⁺CD25⁺ cells, expressed as a percentage of total CD4⁺ cells, were not different between the CY/OVA and ovalbumin mice at day 19 or 26 (Table I, B, and Fig E2, A-E, in the Online Repository at www.jacionline.org). However, CD25⁺ cells may include effector T cells in addition to Treg, so another marker of Treg, CTLA-4, was determined. At day 26, CTLA-4⁺ cells, expressed as a percent of total CD4⁺ T cells, were significantly lower in CY/OVA mice compared with ovalbumin mice (Table I, C, and Fig E2, A-E, in the Online Repository at www.jacionline.org).

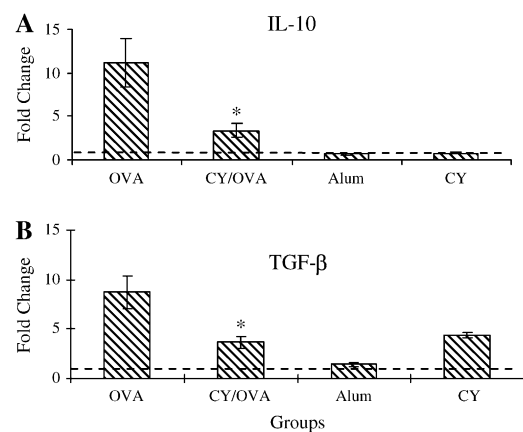


FIG 4. Expression of (A) IL-10 and (B) TGF- β mRNA in lung, determined by quantitative RT-PCR. Data are expressed as fold change, with values from untreated mice defined as 1. There were 6 mice in each group. Data are shown as means $\pm$ SEMs. * P < .05 compared with ovalbumin (OVA) mice. The experiment shown is representative of 2 separate experiments. CY, Cyclophosphamide.

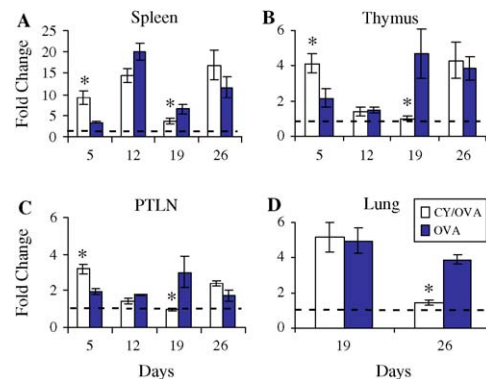


FIG 5. Expression of FoxP3 mRNA by quantitative RT-PCR. Purified CD4⁺ T cells from (A) spleens, (B) thymuses, (C) PTLNs, and (D) lungs were prepared. There were 6 mice in each group. Data are shown as means $\pm$ SEMs. * P < .05 compared with ovalbumin (OVA) mice. The experiment shown is representative of 2 separate experiments. CY, Cyclophosphamide.

IL-10 production was assessed by intracellular staining after *in vitro* stimulation. In cells taken from mice at day 26, IL-10-producing CD4⁺ T cells were significantly lower in CY/OVA mice compared with ovalbumin mice. In the CY/OVA group, IL-10-producing CD4⁺ T cells were significantly reduced after challenge compared with before challenge (Table I, D, and Fig E2, F-J, in the Online Repository at www.jacionline.org). The effects on absolute numbers of these subsets were similar, and the data are presented in this article's Table E2 in the Online Repository at www.jacionline.org.

DISCUSSION

We have demonstrated that administration of cyclophosphamide was followed by increased airway allergic

TABLE I. Flow cytometry analysis of lung cells

A, Absolute number of lung CD4⁺ T cells ($\times 10^3$)			
	CY/OVA	Ovalbumin	Untreated
Day 19	455 $\pm$ 49	638 $\pm$ 89	810 $\pm$ 149
Day 26	402 $\pm$ 44 [†]	904 $\pm$ 55	
B, Lung CD4⁺CD25⁺ cells (% of total CD4⁺ cells)			
	CY/OVA	Ovalbumin	Untreated
Day 19	7.25 $\pm$ 0.81	3.14 $\pm$ 0.71	5.76 $\pm$ 1.82
Day 26	13.8 $\pm$ 2.76	9.06 $\pm$ 0.75	
C, Lung CD4⁺CTLA-4⁺ cells (% of total CD4⁺ cells)			
	CY/OVA	Ovalbumin	Untreated
Day 19	12.1 $\pm$ 1.48	10.1 $\pm$ 0.53	21.6 $\pm$ 2.68
Day 26	9.53 $\pm$ 2.28 [†]	17.3 $\pm$ 2.17	
D, Lung CD4⁺IL-10⁺ cells (% of total CD4⁺ cells after concanavalin A)			
	CY/OVA	Ovalbumin	Untreated
Day 19	36.8 $\pm$ 1.1	29.7 $\pm$ 5.29	17.6 $\pm$ 1.06
Day 26	11.9 $\pm$ 1.22 ^{*†}	18.1 $\pm$ 1.44	

Data are shown as means $\pm$ SEMs. Six mice per group.

* $P < .05$ day 19 versus day 26; [†] $P < .05$ and [‡] $P < .01$ CY/OVA mice versus ovalbumin mice.

inflammation in a mouse model. Features increased by cyclophosphamide included eosinophilia in the lung tissue (Fig 1) and BAL fluid (Fig 2), serum IgE, and production of T_H2 effector cytokines, IL-4 and IL-5 (Fig 3). The features augmented by cyclophosphamide are all consistent with a T_H2 response. Cyclophosphamide is well known to augment T_H1-mediated autoimmune diseases and other T_H1-associated immune responses. By contrast, to our knowledge, our report is the first description of cyclophosphamide exacerbating a model of a T_H2-driven allergic disease, although cyclophosphamide has been shown to increase antigen-induced eosinophilia²³ by increased production of IL-5,²⁷ and antigen-driven IgE production.²⁸ Thus, cyclophosphamide is capable of augmenting both T_H1 and T_H2-mediated immune responses.

In nonobese diabetic mouse models, administration of cyclophosphamide accelerated the onset of autoimmune IDDM. Cyclophosphamide caused an initial drop in total cell numbers in lymphoid organs, followed by rapid reconstitution.²⁹ The acceleration of IDDM by cyclophosphamide is associated with a marked increase in IFN- γ -secreting T cells in the pancreatic islets.³⁰ It is possible that in our study, after treatment with cyclophosphamide and immunization with ovalbumin in alum, the reconstitution of lymphoid organs includes overrepresentation of antigen-specific T_H2 cells, which then leads to pulmonary inflammation after allergen challenge.

The effects of cyclophosphamide may be a result of toxicity of cyclophosphamide to Treg. In a report on tumor-bearing rats, cyclophosphamide decreased the number of CD4⁺CD25⁺ Tregs.¹⁰ In another report, treatment with cyclophosphamide decreased cell numbers in lymphoid organs and reduced the suppressive capacity

of CD4⁺CD25⁺ Treg. Cyclophosphamide enhanced apoptosis of Treg, potentiated loss of homeostatic proliferation of Treg, and downregulated expression of the Treg markers glucocorticoid-induced tumor necrosis factor receptor (GITR) and FoxP3.²⁴ The dose of cyclophosphamide used in this report, 100 mg/kg, was similar to that used in the current experiments, 150 mg/kg.

The transcription factor FoxP3 is a specific marker of naturally occurring CD4⁺CD25⁺ Treg,¹⁸ and our data on FoxP3 expression indicate an effect of cyclophosphamide on these cells (Fig 5), suggesting that repeated treatments with cyclophosphamide may potentiate inflammatory diseases by decreasing the proportion of naturally occurring Treg. In the lungs of cyclophosphamide-treated mice, FoxP3 expression was reduced after challenge, and the reduction of Treg may contribute to the exacerbation of inflammation. Before challenge, lung FoxP3 expression was not reduced. These results suggest that the 2 doses of cyclophosphamide are insufficient to affect the proportion of naturally occurring Treg in the lung, and that allergen challenge is required to elicit a change in local T cells and a reduction in the proportion of naturally occurring Treg.

Treatment with cyclophosphamide was also associated with reduced mRNA levels of the immunosuppressive cytokines IL-10 and TGF- β in the lung after challenge (Fig 4). Furthermore, IL-10-producing CD4⁺ T cells in the lungs of cyclophosphamide-treated mice were significantly reduced after challenge (Table I and Fig E2 in the Online Repository at www.jacionline.org). This result correlates with the data on IL-10 mRNA, although various cell lineages in addition to CD4⁺ T cells are likely to contribute to the total IL-10 mRNA. It is controversial whether naturally occurring Tregs produce IL-10,³¹ but T-regulatory 1 cells, which are IL-10-producing inducible Tregs that do not express FoxP3,^{31,32} could contribute to the IL-10 production in our experiments. Total CD4⁺ cells and CTLA-4⁺CD4⁺ T cells were reduced in the lungs of CY/OVA mice after ovalbumin aerosol challenge (Table I and Fig E2 in the Online Repository at www.jacionline.org). These CTLA-4⁺CD4⁺ T cells might be a subset of regulatory T cells, but surface markers that specifically define regulatory T cells are still elusive. In CD4 T cells, CD25 is a marker of both Treg and activated effector cells. It is possible that the lack of significant change in CD4⁺CD25⁺ cells may be caused by a reduction in Treg combined with an increase in effector T cells. Although our data are consistent with a model in which the effects are mediated by toxicity of cyclophosphamide to Treg, we cannot exclude the possibility that toxicity to other cell types may contribute to the effects of cyclophosphamide.

Cyclophosphamide is well known as a cytotoxic drug and an immunosuppressive agent. By contrast, cyclophosphamide is also able to augment certain immune responses including the eosinophilic response under our experimental protocol. The timing of administration of cyclophosphamide may be critical in determining either immunosuppression or enhanced immune response. Our

study suggests that clinical usage of cyclophosphamide and other chemotherapeutic agents may have undesirable effects on allergic diseases. Two children exhibited improvement of asthma during chemotherapy for leukemia but relapse after completion of therapy.³³ These results suggest that high-dose chemotherapy may inhibit CD4⁺ T-cell-driven diseases, such as allergic asthma, but that this effect may be transient. Our study raises the possibility that allergic inflammatory diseases may be worsened in the recovery phase after treatment with chemotherapeutic agents such as cyclophosphamide.

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