

Alleviating allergic airway diseases by means of short-term administration of IL-2 and dexamethasone

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Background: IL-2 combined with dexamethasone can upregulate regulatory T (Treg) cells, but the mechanism is still under exploration.

Objective: Although previous studies focused on upregulating Treg cells in normal mice, here we investigated whether the IL-2 and dexamethasone combination treatment can upregulate Treg cells in pathological conditions, specifically in alleviating allergic airway disease. We also examined the potential pathway involved in Treg cell upregulation by IL-2 and dexamethasone.

Methods: We evaluated the dose of IL-2 and dexamethasone required to upregulate Treg cells *in vivo* and *in vitro*. We also tested IL-2 and dexamethasone in the intervention of allergic airway disease in a murine model.

Results: We found that administration of 400,000 IU of IL-2 and 0.1 mg of dexamethasone per mouse was effective in upregulating Treg cells, as well as in alleviating allergic airway disease in an established animal model, but this phenomenon disappeared after anti-CD25 antibody administration. We discovered that an *in vitro* low dose of IL-2 can protect Treg cells did not protect CD4⁺CD25⁻ cells from dexamethasone-induced apoptosis by affecting forkhead box O3a phosphorylation through the Akt and serum and glucocorticoid-induced protein kinase pathways.

Conclusions: IL-2/dexamethasone treatment can alleviate existing allergic airway diseases by upregulating Treg cells *in vivo*. A low dose of IL-2 (10⁻⁹ to 10⁻¹¹ mol/L) can protect Treg cells but not CD4⁺CD25⁻ cells from dexamethasone-induced apoptosis *in vitro*, thereby explaining a possible mechanism of increased proportion of Treg cells. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Regulatory T cells, FoxO3a, CD25

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Abbreviations used

BALF: Bronchoalveolar lavage fluid
Bim: bcl-2 Interacting mediator of cell death
CTLA-4: Cytotoxic T lymphocyte-associated antigen 4
FoxO3a: Forkhead box O3a
FoxP3: Forkhead box protein 3
H&E: Hematoxylin and eosin
OVA: Ovalbumin
PAS: Periodic acid-Schiff
SGK: Serum and glucocorticoid-induced protein kinase
Treg: Regulatory T

Allergic airway diseases, such as asthma and allergic rhinitis, are worldwide problems, and their prevalence continues to increase in both developing and developed countries.^{1,2} Recent studies and more current data now support a role for regulatory T (Treg) cells in alleviating allergic airway diseases.³⁻⁶ Although many investigators tried to alleviate asthma by infusion of Treg cells,^{3,5,6} a few have attempted to upregulate Treg cells with such treatments as IL-2/IL-2 antibody⁴ and 1, 25-(OH)₂ VitD3.^{7,8} Although these studies showed successful upregulation of Treg cells, they were usually used to prevent disease.⁴ Steroids can also upregulate Treg cells, but the effect is very short lived, and long-term administration results in many side effects.⁹

Short-term simultaneous administration of dexamethasone and IL-2 have been shown to markedly expand the numbers of functional suppressive Foxp3⁺CD4⁺CD25⁺ T cells in murine peripheral lymphoid tissues.⁹ Up to now, however, no one has fully determined the effective therapeutic regimen and mechanism of using IL-2 plus dexamethasone to treat disease.

In this study we determined an optimal dose of IL-2 and dexamethasone per mouse that could maximally and effectively upregulate Treg cells and investigated the effect of this regimen in alleviating ovalbumin (OVA)-induced allergic airway disease in mice. We found that Treg cells could be upregulated and lung inflammation could be alleviated with IL-2/dexamethasone treatment both in diseased and healthy mice. We further clarified that the effects of IL-2/dexamethasone treatment disappeared after anti-CD25 antibody administration. We also explored the mechanism by which IL-2 plus dexamethasone might upregulate Treg cells. We investigated the role of forkhead box O3a (FoxO3a) phosphorylation and the selective activation of IL-2/IL-2 receptor pathways in Treg cell survival. Therefore our study provides evidence that the combination of IL-2 and dexamethasone might prove to be an effective method for *in vivo* expansion of Treg cells, which could potentially be used for the treatment of allergies and other types of inflammatory diseases.

METHODS

Animals and antibodies

Female BALB/c mice (6–8 weeks) were obtained from the animal department of the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, and were maintained in pathogen-free conditions. All animal experiments were performed according to institutional and state guidelines. The animal protocol was approved by the Institutional Animal Use Committee of the Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences).

Immunization and intervention

Mice were immunized intraperitoneally with OVA (20 µg; Sigma-Aldrich, St Louis, Mo) plus aluminum hydroxide (Alum; Pierce, Rockford, Ill). This was followed by 40 µg of OVA in 40 µL of PBS administered intranasally at the indicated time.¹⁰ Recombinant human IL-2 (Huaxin Corp, Shanghai, China) and dexamethasone (Xianju Corp, Zhejiang, China) were injected intraperitoneally at various doses at the indicated time. All mice were killed 2 days after the last OVA challenge.

For *in vivo* CD25 depletion, mice were sensitized, and one group received 150 µg of functional grade purified anti-mouse CD25 (PC61.5; eBioscience, San Diego, Calif) intravenously at the indicated time.

IL-2/dexamethasone treatment and flow cytometry

IL-2 and dexamethasone were injected intraperitoneally for 3 days in 2 stages: (1) before the beginning of the OVA-priming stage and (2) after the OVA-priming stage and before the OVA challenge. Finally, after the mice were challenged with OVA, intranasal OVA challenge was made, if needed. Splenocytes were analyzed by means of flow cytometry 1 day after the drug intervention, and lympho nodes and lung tissues were analyzed by means of flow cytometry 1 day after OVA boosting. Cells were collected and stained with fluorescein isothiocyanate-conjugated CD4, phycoerythrin (Pe)-cy5-conjugated forkhead box protein 3 (FoxP3), and phycoerythrin-conjugated cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) antibody or isotype control.

Mice were divided into 4 groups and injected with dexamethasone (0.1 mg) alone, IL-2 (400,000 IU) combined with PBS, dexamethasone (0.1 mg per mouse), or IL-2 (1,000,000 IU) combined with dexamethasone (0.1 mg) to evaluate apoptosis of Treg cells after IL-2 and dexamethasone treatment *in vivo*. Eight hours after the treatment, lymphocytes were collected, washed, and resuspended in binding buffer and then incubated with fluorescein isothiocyanate-labeled Annexin V (Sigma) and allophycocyanin (APC)-conjugated CD4 (eBioscience) for 15 minutes in the dark at room temperature. Subsequently, the surface-labeled cells were washed, fixed, permeabilized, and then stained with the phycoerythrin anti-mouse Foxp3 staining set (eBioscience) and analyzed by means of flow cytometry.

Suppression assays

Initially, a monoclonal anti-mouse CD3 antibody (R&D Corp) was reconstituted at 1 µg/mL. A 96-well plate was coated with 50 µL per well of the CD3 antibody overnight at 4°C. Splenocytes (including Treg cells) from pretreated mice were incubated in the CD3-coated plate or in a CD3-uncoated plate for 6 days, after which cell proliferation was determined by measuring cell numbers with flow cytometry.

Collection of bronchoalveolar lavage fluid

Animals were killed and treated as previously described.¹¹ The cytokine levels in bronchoalveolar lavage fluid (BALF) were measured by using IL-4, IL-5, and IFN-γ ELISA kits (R&D Corp, Minneapolis, Minn).

Lung histology

Lungs were fixed in 10% formalin and stained with hematoxylin and eosin (H&E; Merck & Co, Inc, Whitehouse Station, NJ) or with Alcian blue/periodic acid-Schiff (PAS; Sigma-Aldrich). All slides were examined in a random blind fashion by 2 independent investigators.

Purification of cells

Untreated murine spleens were aseptically removed. CD4⁺CD25⁺ and CD4⁺CD25[−] T cells were isolated from the splenocytes by using the CD4⁺CD25⁺ murine regulatory T Cell Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany). The purity of CD4⁺CD25⁺ T cells was greater than 90%, and that of CD4⁺CD25[−] T cells was greater than 95%. Purified cells were suspended and cultured in RPMI 1640 with 10% FBS (Hyclone, Logan, Utah) containing 100 IU/mL penicillin and 100 µg/mL streptomycin.

Western blotting

After harvesting, the expression of various proteins in the cells were detected by using antibodies from Cell Signaling (Danvers, Mass), as previously described.¹²

Induction of apoptosis

Dexamethasone (Sigma-Aldrich) was dissolved according to the method described in our previous study.¹² Lymphocytes from various groups were pretreated as indicated with recombinant murine IL-2 (Sigma-Aldrich) and then with dexamethasone or control reagents. After a 12-hour incubation period, the lymphocytes were harvested for analysis by means of Western blotting and for determining apoptosis levels, as previously described.¹²

Susceptibility of CD4⁺CD25⁺ T cells and CD4⁺CD25[−] T cells to apoptosis after Akt inhibitor treatment

Sorted CD4⁺CD25⁺ and CD4⁺CD25[−] T cells were cultured with or without an Akt inhibitor (3 µmol/L; Calbiochem, San Diego, Calif) for 2 hours, and then phospho-FoxO3a expression was detected. Akt inhibitor-pretreated cells were incubated with IL-2 and dexamethasone for 6 hours, and then apoptosis and bcl-2 interacting mediator of cell death (Bim) expression were measured.

Statistics

The 2-tailed Student *t* test was used to evaluate differences between groups. *P* values of less than .05 were considered statistically significant.

RESULTS

Different doses of IL-2 combined with dexamethasone can differentially upregulate Treg cells

Chen et al⁹ previously reported that IL-2 at a dose of 300,000 IU plus dexamethasone at 0.1 mg per mouse could upregulate Treg cells, but it remained uncertain whether these doses of IL-2 and dexamethasone would have an optimal effect on Treg cell upregulation. In our study, using a fixed 0.1-mg dose of dexamethasone per mouse, we were able to find that a corresponding dose of 400,000 IU of IL-2 per mouse was optimal for Treg cell upregulation (*P* < .05; see Fig E1, A, in this article's Online Repository at www.jacionline.org). However, when we used a fixed dose of 400,000 IU of IL-2 per mouse, we did not observe significant differences in Treg cell upregulation when administered with dexamethasone at doses of 0.1, 0.2, or 0.3 mg (*P* > .05, see Fig E1, B). Therefore to minimize any glucocorticoid-induced side effects, we selected 400,000 IU of IL-2 and 0.1 mg of dexamethasone per mouse as the optimal dose. We identified Treg cells based on expression of CD4 and FoxP3.¹³ In our previous study we had demonstrated that these Treg cells were inhibitory.¹⁴ Because the levels of CTLA-4 can reflect the inhibitory abilities of Treg cells,¹⁵ we also detected CTLA-4⁺FoxP3⁺ cells in all CD4-gated cells.

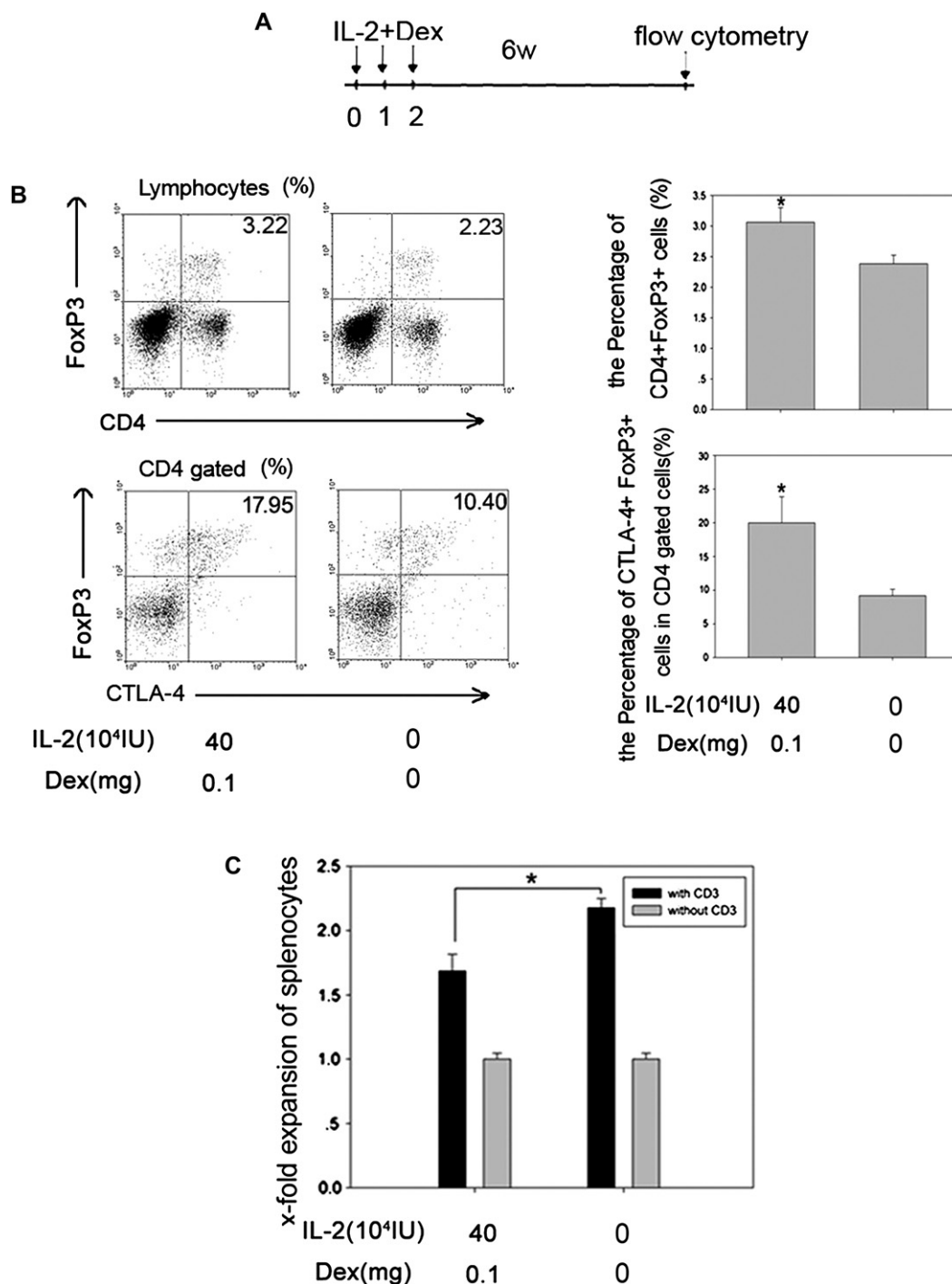


FIG 1. Detection of Treg cells 6 weeks after IL-2 and dexamethasone (Dex) treatment. **A**, Timeline of drug intervention and flow cytometric analysis. **B** and **C**, Detection of Treg cell composition, CTLA-4 expression (Fig 1, **B**), and the proliferation of splenocytes (Fig 1, **C**) 6 weeks after administration of IL-2 plus dexamethasone. Data are presented as means $\pm$ SDs ($n = 3$ per group). * $P < .05$.

IL-2 combined with dexamethasone can upregulate Treg cells at least for 6 weeks

BALB/c mice were administered 400,000 IU of IL-2 and 0.1 mg of dexamethasone per mouse or PBS for 3 days. After 6 weeks, all mice were killed, and Treg cells were detected by means of flow cytometry (Fig 1). We found that the IL-2 plus dexamethasone group had a higher proportion of CD4⁺FoxP3⁺ cells ($P < .05$).

Additionally, upregulation of Treg cells by IL-2 plus dexamethasone could occur at different stages of OVA immunization, even after the OVA-priming (see Fig 2, **A**, in this article's Online Repository at www.jacionline.org) and OVA-challenge stages (see Fig E2, **B**). We also found after IL-2 and dexamethasone administration (no antigen sensitization) that the proportion of Treg cells has increased in lymph nodes, although not in lung tissues (data

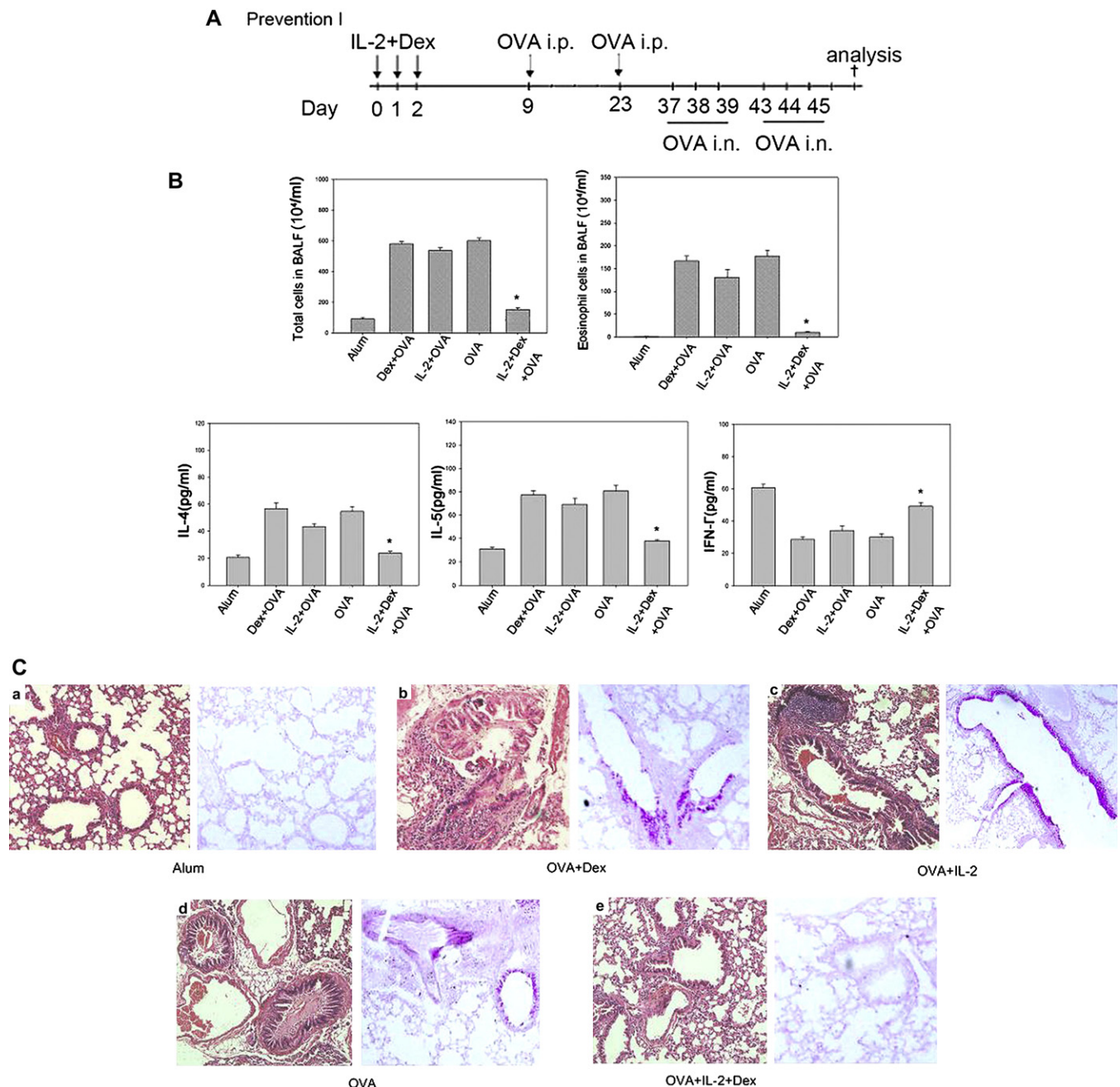


FIG 2. Manifestations of allergic airway disease after IL-2 and dexamethasone (Dex) administered before OVA priming. **A**, IL-2 and dexamethasone were administered for 3 days before OVA priming. **B**, Measurements of lymphocyte cell counts, cytokines in BALF, and lung inflammation after OVA challenge. **C**, Images of lung sections ($\times 100$ magnification). Left, H&E staining; right, PAS staining. Data are presented as means $\pm$ SDs ($n = 6$ per group). * $P < .05$. i.n., Intranasal; i.p., intraperitoneal.

not shown). However, if the mice received IL-2 and dexamethasone administration and OVA challenge, Treg cells were upregulated both in lymph nodes and in lung cells (see Fig E2, C and D).

IL-2 combined with dexamethasone can prevent airway inflammation in OVA-immunized BALB/c mice

We administered 400,000 IU of IL-2 plus 0.1 mg of dexamethasone per mouse for 3 days 1 week before the beginning of OVA

priming. Two days after the last OVA challenge, we evaluated the murine BALF and found that total cell count, eosinophil cell count, and T_H2 cytokine production were significantly decreased in the IL-2 plus dexamethasone group. Additionally, lung inflammation (hematoxylin and eosin [H&E] stain, $\times 100$ magnification) and mucus production (PAS stain, $\times 100$ magnification) were also decreased in the IL-2 plus dexamethasone group. Importantly, mice receiving either IL-2 or dexamethasone alone did not have a similar response (Fig 2), suggesting that the combination of these agents was responsible for the observed effects.

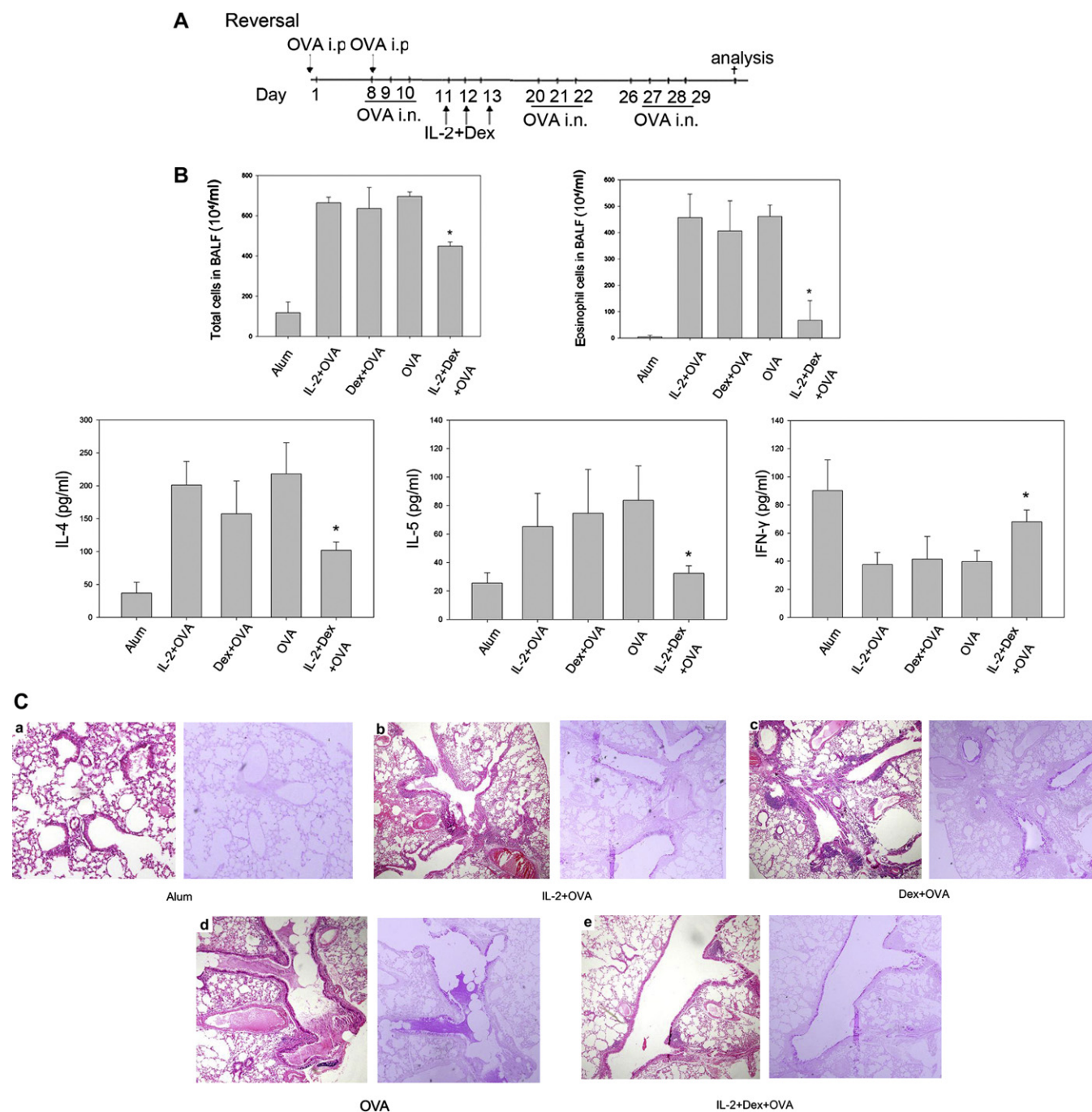


FIG 3. Manifestations of allergic airway disease after IL-2 and dexamethasone (*Dex*) were administered after OVA challenge. **A**, IL-2 and dexamethasone were administered for 3 days after OVA challenge. **B**, Analysis of lymphocyte cell counts, cytokines in BALF, and lung inflammation after OVA rechallenge. **C**, Images of lung sections ($\times 40$ magnification). *Left*, H&E staining; *right*, PAS staining. Data are presented as means $\pm$ SDs ($n = 6$ per group). * $P < .05$. *i.n.*, Intranasal; *i.p.*, intraperitoneal.

In the group administered 400,000 IU of IL-2 plus 0.1 mg of dexamethasone per mouse for 3 days after OVA priming but before OVA boosting, we analyzed BALF 2 days after the last OVA challenge and found that total cell count, eosinophil cell count, and T_H2 cytokine production were significantly decreased in the IL-2 plus dexamethasone group. Lung inflammation (H&E stain, $\times 40$ magnification) and mucus production (PAS stain, $\times 40$ magnification) were also diminished in the IL-2 plus

dexamethasone group. There were no statistical differences between the cellular responses of the other intervention groups (see Fig E3 in this article's Online Repository at www.jacionline.org).

In the reverse protocol mice were injected with OVA intraperitoneally on day 1 and challenged with OVA on days 8, 9, and 10. On days 11, 12, and 13, the mice were again injected with 400,000 IU of IL-2, 0.1 mg of dexamethasone, or PBS intraperitoneally.

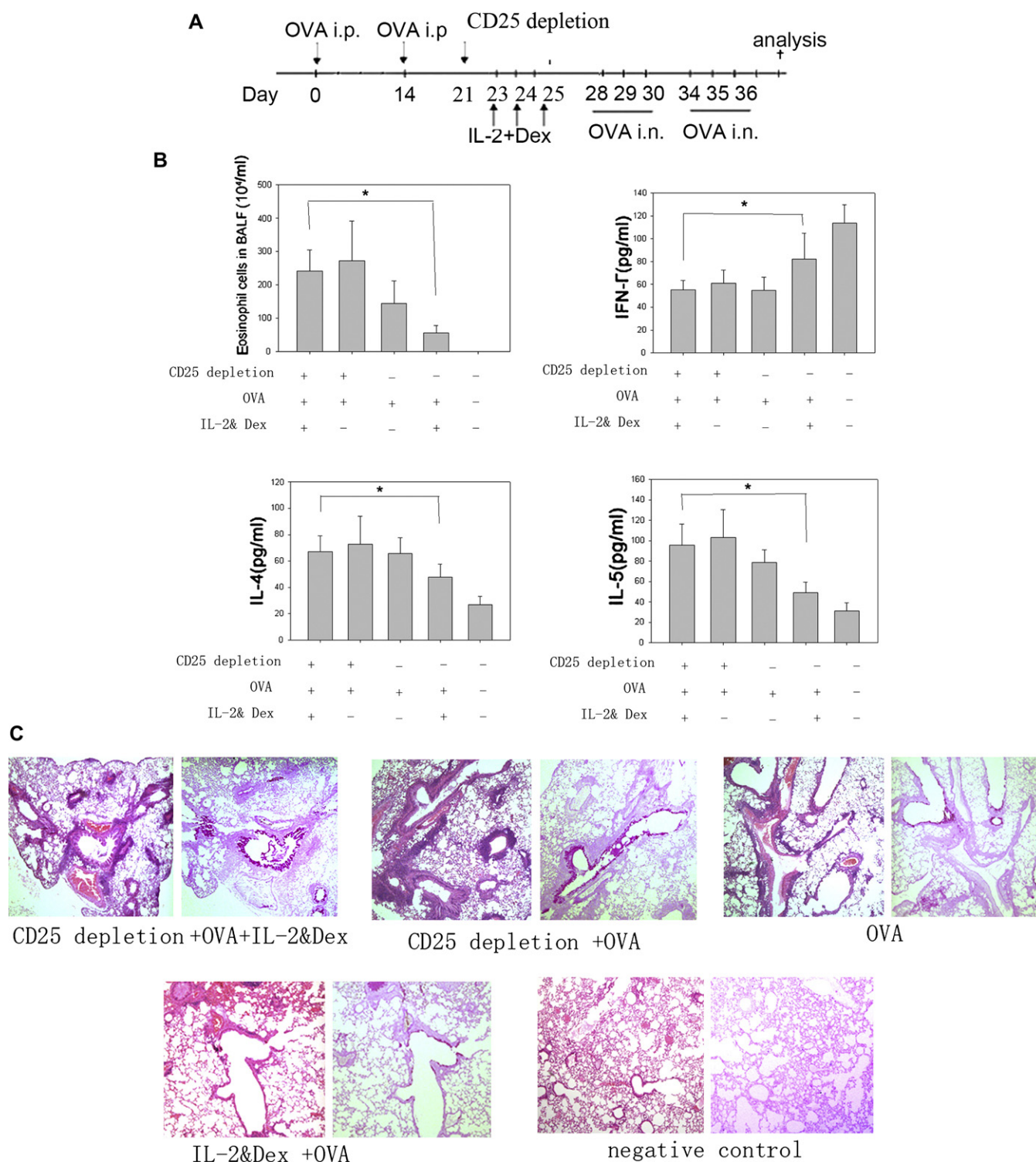


FIG 4. Manifestations of allergic airway disease after Treg cell depletion and medical intervention. **A**, IL-2 and dexamethasone (*Dex*) were administered after anti-CD25 antibody administration. **B**, Analysis of eosinophil counts and cytokines in BALF after OVA challenge. **C**, Images of lung sections ($\times 40$ magnification). *Left*, H&E staining; *right*, PAS staining. Data are presented as means $\pm$ SDs ($n = 4$ per group). * $P < .05$. *i.n.*, Intranasal; *i.p.*, intraperitoneal.

One week later, the mice were rechallenged with OVA, and 2 days after the last OVA challenge, BALF was evaluated. We found that treatment with IL-2 plus dexamethasone alleviated lung inflammation, even after OVA challenge. Total cell counts in

BALF were lower, especially the significantly decreased eosinophil cell count, in the IL-2 plus dexamethasone group than in the allergic airway disease group. Lung inflammation (H&E stain, $\times 40$ magnification) and mucus production (PAS stain, $\times 40$

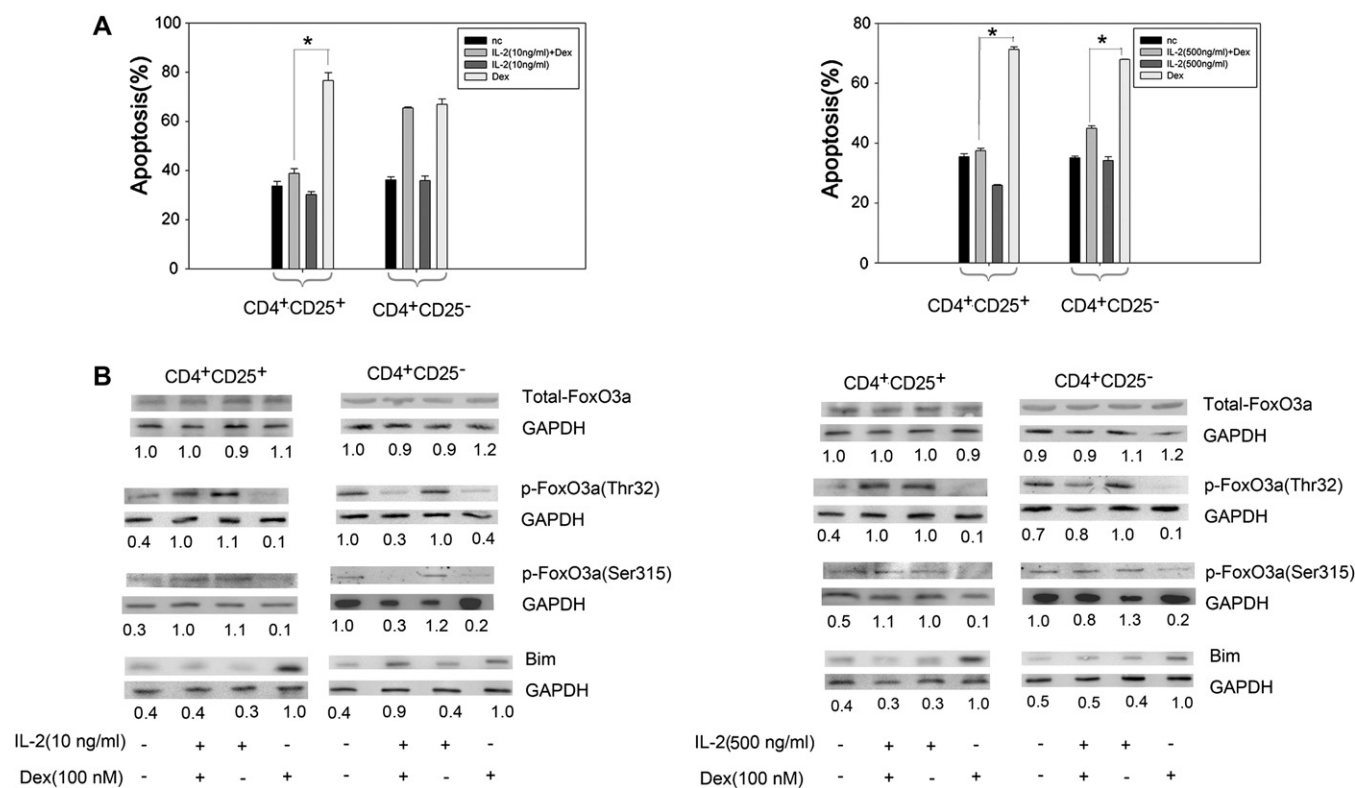


FIG 5. Apoptosis and Western blot analysis of CD4⁺CD25⁺ and CD4⁺CD25⁻ cells treated *in vitro*. **A**, Apoptosis and expression of FoxO3a, phospho-FoxO3a, and Bim in the presence of 10 ng/mL IL-2 or as indicated. **B**, Apoptosis and expression of FoxO3a, phospho-FoxO3a, and Bim in the presence of 500 ng/mL IL-2 or as indicated. The apoptosis analysis was repeated 3 times, and all Western blots were repeated 2 to 3 times. Data are presented as means $\pm$ SDs. * $P < .05$. Dex, Dexamethasone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

magnification) were slightly diminished by this combination treatment (Fig 3).

Mice also received anti-CD25 treatment after OVA sensitization and then IL-2 plus dexamethasone administration and OVA intranasal challenge (Fig 4, A). We found that after Treg cell depletion, treatment with IL-2 plus dexamethasone could not upregulate Treg cells (see Fig E4 in this article's Online Repository at www.jacionline.org) and alleviate allergic airway diseases again. Compared with the IL-2/dexamethasone group, eosinophil counts in BALF and T_H2 cytokine production increased. Lung inflammation (H&E stain, $\times 40$ magnification) and mucus production (PAS stain, $\times 40$ magnification) were exacerbated after anti-CD25 antibody administration (Fig 4, B and C).

CD4⁺CD25⁺ cells and CD4⁺CD25⁻ cells have different susceptibilities to dexamethasone-induced apoptosis in the presence of IL-2 *in vivo* and *in vitro*

Treg cells were protected from dexamethasone-induced apoptosis at the dose of 400,000 IU of IL-2 per mouse *in vivo*. When the dose of IL-2 was increased to 1,000,000 IU per mouse, CD4⁺FoxP3⁺ cells were also protected from dexamethasone-induced apoptosis, whereas apoptosis of Treg cells increased, causing the ratio of Treg cells to be even lower than with a relatively low dose of IL-2 (see Fig E5).

When untreated murine splenocytes were sorted for CD4⁺CD25⁺ (mainly Treg cells) and CD4⁺CD25⁻ cells and incubated for 12 hours *in vitro* with the indicated dose of IL-2 plus dexamethasone, IL-2 alone, or dexamethasone alone, we found that CD4⁺CD25⁻ cells were more susceptible to dexamethasone (100 nmol/L)-induced apoptosis than CD4⁺CD25⁺ cells in the presence of 10 ng/mL IL-2; that is, a relatively low dose of IL-2 could protect CD4⁺CD25⁺ cells but not CD4⁺CD25⁻ cells from dexamethasone-induced apoptosis. However, when the IL-2 concentration was increased to 500 ng/mL, dexamethasone-induced apoptosis of the CD4⁺CD25⁻ cells was significantly reduced (Fig 5, A), suggesting that the ratio of CD4⁺CD25⁺/CD4⁺CD25⁻ cells can be upregulated in the presence of a relatively low dose of IL-2 plus dexamethasone.

Expression of phospho-FoxO3a is negatively correlated with dexamethasone-induced CD4⁺CD25⁺ and CD4⁺CD25⁻ cell apoptosis

Expression of phospho-FoxO3a was negatively correlated with the level of dexamethasone-induced apoptosis of CD4⁺CD25⁺ and CD4⁺CD25⁻ cells, which is consistent with the results of our previous study.¹² Therefore we next explored whether IL-2 could overcome dexamethasone-induced CD4⁺CD25⁺ cell apoptosis through affecting FoxO3a. CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were sorted and incubated with the indicated dose of IL-2 plus dexamethasone. In the presence of 10 ng/mL

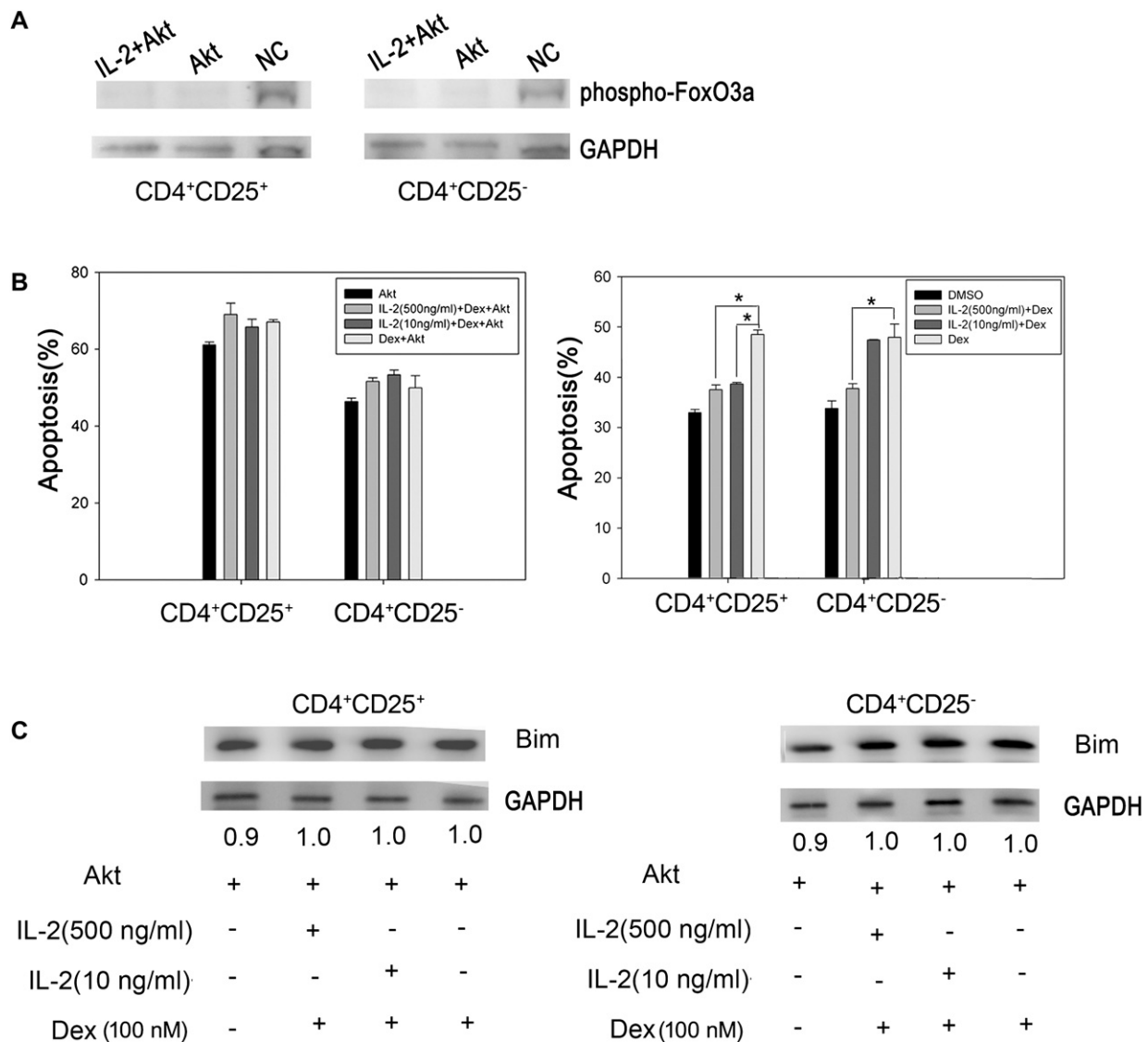


FIG 6. Apoptosis and Western blot analyses of CD4⁺CD25⁺ and CD4⁺CD25⁻ cells after Akt inhibitor treatment. **A**, Expression of phospho-FoxO3a (Thr32) after pretreatment with an Akt inhibitor. **B**, Levels of the apoptotic corresponding cells in the presence of the indicated dose of treatment. **C**, Expressions of Bim in the corresponding cells in the presence of the indicated dose of treatment. Similar results were obtained in 3 independent experiments. Data are presented as means $\pm$ SDs. * $P < .05$. Akt, With Akt inhibitor; Dex, dexamethasone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

IL-2 and 100 nmol/L dexamethasone, we found that CD4⁺CD25⁺ cells had a higher expression of phospho-FoxO3a (Ser315, Thr32), a lower level of apoptosis, and lower expression of Bim than did CD4⁺CD25⁻ cells. Meanwhile, in the presence of 500 ng/mL IL-2, dexamethasone-induced apoptosis and Bim expression of CD4⁺CD25⁻ cells decreased and expression of phospho-FoxO3a increased (Fig 5, B).

There are 2 phosphorylation kinases (serum and glucocorticoid-induced protein kinase [SGK] and Akt) in the phosphoinositide 3-kinase pathway that can phosphorylate FoxO3a. The Akt phosphorylation sites are Ser253 and Thr32, and the SGK phosphorylation sites are Ser315 and Thr32.¹⁶ In our study we found phospho-FoxO3a (Ser315 and Thr32) bands but no clear Ser253 bands after treatment with IL-2 plus dexamethasone.

Inhibition of Akt decreased phospho-FoxO3a expression in CD4⁺CD25⁺ cells and CD4⁺CD25⁻ cells

Because we did not have sufficient evidence from the experiment above to suggest involvement of Akt in the phosphorylation of FoxO3a, we next used an Akt inhibitor (Akt-IV inhibitor, 3 μ mol/L) to pretreat for 2 hours and splenocytes from BALB/c mice that had been sorted into CD4⁺CD25⁺ and CD4⁺CD25⁻ cell populations, as above. Our results showed that FoxO3a phosphorylation was significantly decreased both in CD4⁺CD25⁺ cells and in CD4⁺CD25⁻ cells with Akt inhibitor (Fig 6, A). These pretreated cells were also incubated with IL-2 at 10 or 500 ng/mL along with the Akt-IV inhibitors and dexamethasone (100 nmol/L) and then analyzed for expression of apoptosis and Bim. However, the different concentration of

IL-2 did not cause any significant difference in the level of apoptosis or expression of Bim between the CD4⁺CD25⁺ and CD4⁺CD25⁻ cell subsets (Fig 6, B and C). Together, these data suggested that Akt is involved in the phosphorylation of FoxO3a.

DISCUSSION

Allergic airway diseases have become a worldwide problem. Steroids, β_2 -agonists, and allergen-specific immunotherapy all have many side effects. A number of animal and clinical studies support the manipulation of Treg cells to control airway inflammation and airway hyperresponsiveness through the inhibition of antigen-presenting cells, T_H2 cells, mast cells, eosinophils, and basophils,^{5,6,17} but these previous studies required *in vitro* isolation of Treg cells,³ which restricted their use. Some studies attempted to upregulate Treg cells by means of drug treatment, but all these interventions were tested in the prevention of disease,⁴ whereas there were no reports of whether the upregulation effect could be induced in pathological conditions.

In our study we found that IL-2 at the dose of 400,000 IU combined with dexamethasone at 0.1 mg per mouse was most effective for Treg cell upregulation, an effect that was maintained for at least 6 weeks. Our protocol could upregulate Treg cells in both normal and pathological conditions. We also conducted a series of tests after OVA boosting and found that, irrespective of whether treatment was administered before or after the OVA-priming stage or after the OVA-challenge stage, indices such as lymphocyte count, cytokine counts in BALF, and lung inflammation were decreased. We also found that the alleviation of allergic airway diseases and upregulation of Treg cells by IL-2 plus dexamethasone had disappeared after Treg cell depletion, which again proved that treatment with IL-2 plus dexamethasone could alleviate allergic airway diseases mediated by upregulating Treg cells.

A potential explanation for the upregulation of Treg cells is the differential response of CD4⁺CD25⁺ and CD4⁺CD25⁻ cells to dexamethasone in the presence of IL-2. With IL-2 at a concentration of 10 ng/mL (equal to 6×10^{-10} mol/L) *in vitro*, CD4⁺CD25⁺ cells had a higher expression level of phospho-FoxO3a and were more resistant to dexamethasone-induced apoptosis than CD4⁺CD25⁻ cells. However, with IL-2 at a higher concentration of 500 ng/mL (equal to 3×10^{-8} mol/L), CD4⁺CD25⁻ cells had a higher phospho-FoxO3a expression and were less susceptible to dexamethasone-induced apoptosis. We also found the same change *in vivo* in that IL-2 could protect Treg cells from dexamethasone-induced apoptosis at the dose of 400,000 IU per mouse, and IL-2 could also protect CD4⁺FoxP3⁻ cells at the dose of 1,000,000 IU per mouse (high dose), whereas the apoptosis rate of Treg cells increased, suggesting that there might be activation-induced cell death in the Treg cells. We also found that Akt and SGK are both involved in FoxO3a phosphorylation; that is, at a low dose of IL-2, Akt and SGK pathways could be activated in CD4⁺CD25⁺ cells but not in CD4⁺CD25⁻ cells, resulting in phosphorylation of FoxO3a and diminished dexamethasone-induced apoptosis. As the concentration of IL-2 increased, the selective activation effects disappeared, and CD4⁺CD25⁻ cells could also be protected from dexamethasone-induced apoptosis. These results were consistent with the *in vivo* results, where 400,000 IU of IL-2 and 0.1 mg of dexamethasone per mouse upregulated a higher proportion of

Treg cells than 500,000 IU of IL-2 and 0.1 mg of dexamethasone per mouse.

The different responses of CD4⁺CD25⁺ and CD4⁺CD25⁻ cells to IL-2 might be related to the expression of CD25 (IL-2 receptor α chain). CD25 binds IL-2 at a concentration of 10^{-11} mol/L.¹⁸ Because CD25⁻ cells express the IL-2 receptor β and γ chains, which are activated at a higher concentration of IL-2 at 10^{-9} mol/L,¹⁹ we hypothesize that if the concentration of IL-2 can be maintained at between 10^{-9} and 10^{-11} mol/L, the IL-2/IL-2 receptor pathway of CD4⁺CD25⁺ cells, but not CD4⁺CD25⁻ cells, can be activated, followed by activation of Akt and SGK, which can phosphorylate FoxO3a. Dephosphorylated FoxO3a is necessary for dexamethasone-induced apoptosis; therefore IL-2/IL-2 receptor activation can protect cells from dexamethasone-induced apoptosis (see Fig E6 in this article's Online Repository at www.jacionline.org), which might partially explain the upregulation of Treg cells by IL-2 plus dexamethasone. However, our hypothesis can only explain the upregulation of natural Treg cells in healthy mice, whereas upregulation of Treg cells in antigen-sensitized or T cell-activated mice still needs further exploration. These antigen-specific Treg cells might be more inhibitory and make more sense.

In our study we can only explain the upregulation of Treg cells from different apoptosis rates of lymphocytes, but there might be other reasons, such as a different expansion rate of Treg cells and other effector T cells, which needs further study. Matthew et al²⁰ showed that signal transducer and activator of transcription 5 can specifically promote FoxP3 promoter activity in CD4⁺CD25⁺ Treg cells. We hypothesize that the appropriate dose of IL-2 can induce IL-2 receptor activation and Janus kinase/signal transducer and activator of transcription pathway activation, ultimately upregulating FoxP3 gene expression.²¹

We found that Treg cells upregulated by IL-2 and dexamethasone administration were different from those upregulated by dexamethasone only in mice and that the drug combination could upregulate Treg cells for at least 6 weeks. Therefore our protocol of administering drugs over a short time period can apparently induce a prolonged upregulation effect on Treg cells. If such an effect could be observed in the clinical setting with a similar protocol, it could greatly benefit patients with allergic airway diseases.

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Key message

- Treg cells can be upregulated by an appropriate dose of IL-2 and dexamethasone, thereby resulting in the alleviation of allergic airway diseases.

REFERENCES

1. Sears MR. Epidemiology of childhood asthma. *Lancet* 1997;350:1015-20.
2. Wills-Karp M, Santeliz J, Karp CL. The germless theory of allergic disease: revisiting the hygiene hypothesis. *Nat Rev Immunol* 2001;1:69-75.
3. Wilson MS, Taylor MD, Balic A. Suppression of allergic airway inflammation by helminth-induced regulatory T cells. *J Exp Med* 2005;202:1199-212.
4. Wilson MS, Pesce JT, Ramalingam TR, Thompson RW, Cheever A, Wynn TA. Suppression of murine allergic airway disease by IL-2: anti-IL-2 monoclonal antibody-induced regulatory T cells. *J Immunol* 2008;181:6942-54.

5. Kearley J, Barker JE, Robinson DS, Lloyd CM. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med* 2005;202:1539-47.
6. Hadeiba H, Locksley RM. Lung CD25+ CD4+ regulatory T cells suppress type 2 immune responses but not bronchial hyperreactivity. *J Immunol* 2003;170:5502-10.
7. Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF, et al. In vitro generation of interleukin 10-producing regulatory CD4+ T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med* 2002;195:603.
8. Jeffery LE, Burke F, Mura M, Zheng Y, Qureshi OS, Hewison M, et al. 1,25-Dihydroxyvitamin D3 and IL-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3. *J Immunol* 2009;183:5458-67.
9. Chen X, Oppenheim JJ, Winkler-Pickett RT, Ortaldo JR, Howard OMZ. Glucocorticoid amplifies IL-2-dependent expansion of functional FoxP3+CD4+CD25+ T regulatory cells in vivo and enhances their capacity to suppress EAE. *Eur J Immunol* 2006;36:2139-49.
10. Polte T. CD137-mediated immunotherapy for allergic asthma. *J Clin Invest* 2006;116:1025-36.
11. Chuang Y-H, Suen J-L, Chiang B-L. Fas-ligand-expressing adenovirus-transfected dendritic cells decrease allergen-specific T cells and airway inflammation in a murine model of asthma. *J Mol Med* 2006;84:595-603.
12. Ma J, Xie Y, Shi Y, Qin W. Glucocorticoid-induced apoptosis requires FoxO3a activity. *Biochem Biophys Res Commun* 2008;377:894-8.
13. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003;4:330-6.
14. Yanhui Xie, Min Wu. Glucocorticoid amplifies IL-2-induced expansion of CD4+CD25+FOXP3+ regulatory T cells in vivo selectively and suppresses graft versus host disease after allogeneic lymphocyte transplantation. *Acta Biochim Biophys Sin* 2009;41:781-91.
15. Tang AL, Teijaro JR, Njau MN. CTLA4 expression is an indicator and regulator of steady-state CD4+FoxP3+ T cell homeostasis. *J Immunol* 2008;181:1806-13.
16. Brunet A, Park J, Tran H. Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). *Mol Cell Biol* 2001;21:952-6.
17. Cottrez F, Hurst SD, Coffman RL, Groux HS. T regulatory cells 1 inhibit a Th2-specific response in vivo. *J Immunol* 2000;165:4848-53.
18. Tamang DL, Redelman D, Alves BN. Induction of granzyme B and T cell cytotoxic capacity by IL-2 or IL-15 without antigens: multiclonal responses that are extremely lytic if triggered and short-lived after cytokine withdrawal. *Cytokine* 2006;36:148-59.
19. Yamagiwa S, Gray JD, Hashimoto S. A role for TGF- β in the generation and expansion of CD4+CD25+ regulatory T cells from human peripheral blood. *J Immunol* 2001;166:7282-9.
20. Burchill MA, Yang J, Vogtenhuber C, Blazar BR, Farrar MA. IL-2 receptor β -dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *J Immunol* 2007;178:280-90.
21. Murray PJ. The JAK-STAT signaling pathway: input and output integration. *J Immunol* 2007;178:2623-9.

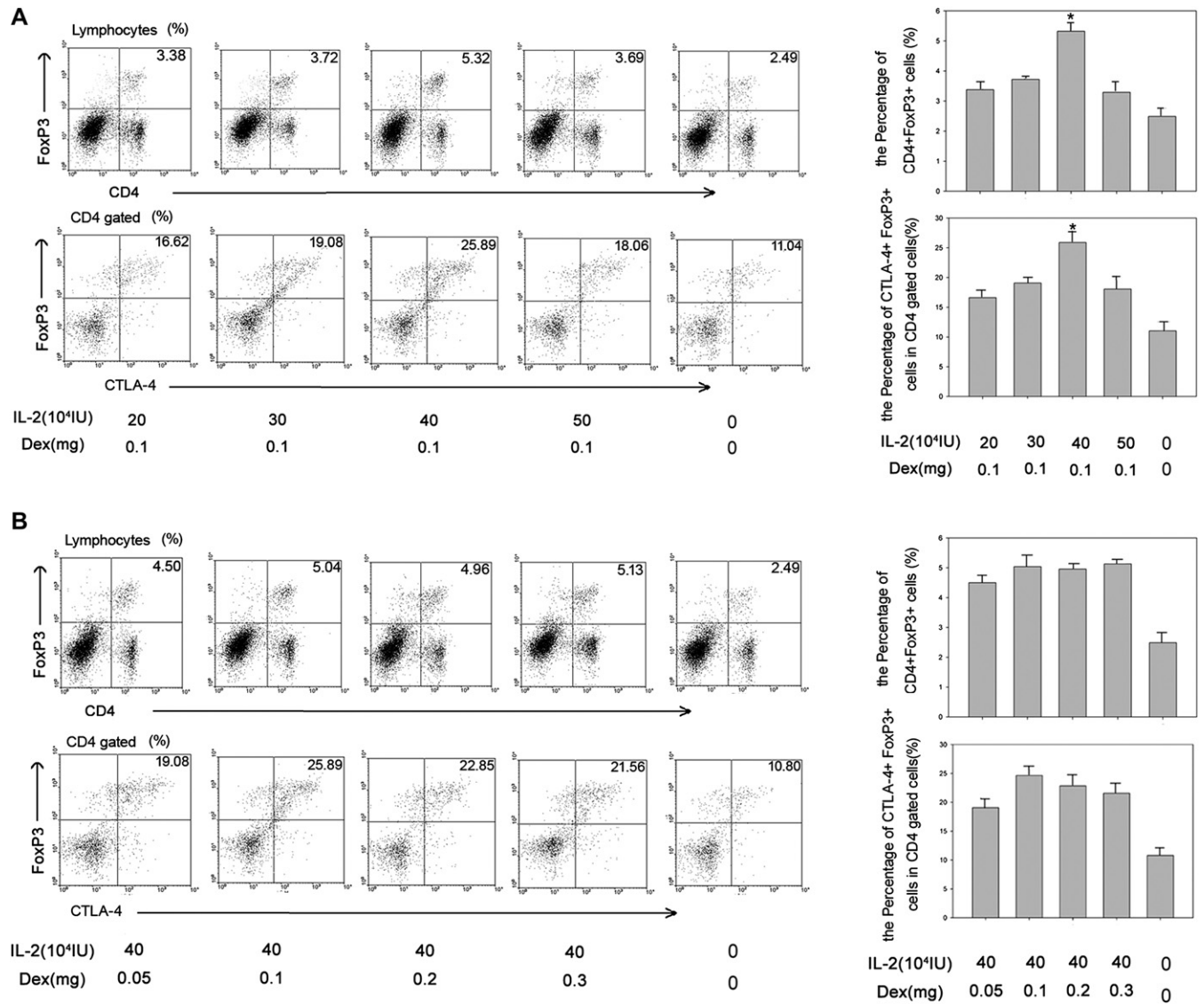


FIG E1. Upregulation of Treg cells by various doses of IL-2 combined with dexamethasone. Treg cell composition and CTLA-4 expression by means of flow cytometry 1 day after administration of different doses of IL-2 and 0.1 mg of dexamethasone (*Dex*) for 3 days (**A**) and different doses of dexamethasone plus 400,000 IU of IL-2 for 3 days (**B**). Data are presented as means $\pm$ SDs ($n = 3$ per group). * $P < .05$.

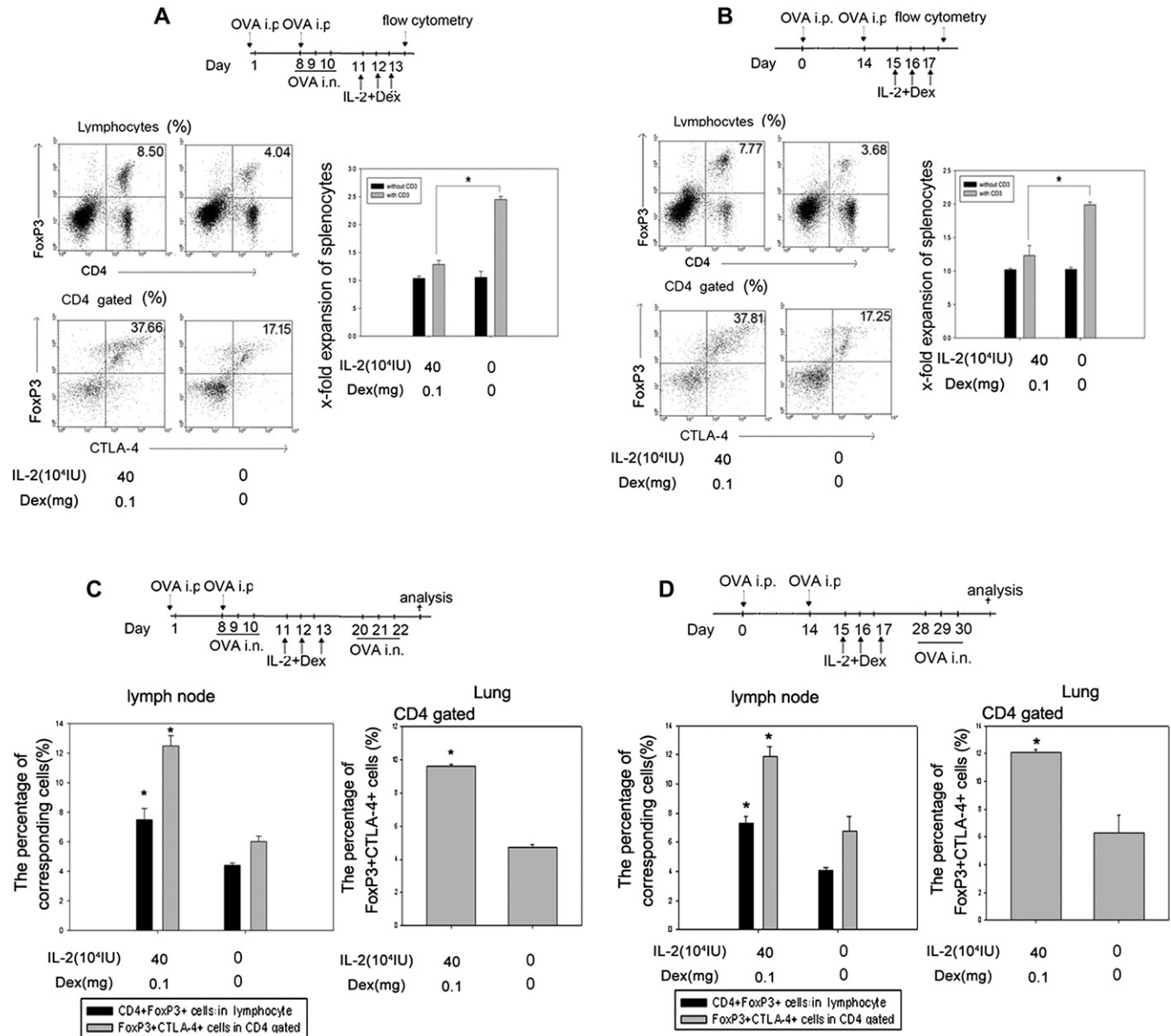


FIG E2. Treg cell analysis after different intervention with different medicines. **A** and **B**, Detection of Treg cells and the proliferation of splenocytes after administration of IL-2 plus dexamethasone (*Dex*) after OVA challenge (Fig E2, **A**) and IL-2 plus dexamethasone after OVA sensitization (Fig E2, **B**). **C** and **D**, Detection of Treg cells in lympho nodes and lung tissues (Fig E2, **C**) and after corresponding intervention (Fig E2, **D**). Data are presented as means $\pm$ SDs ($n = 3$ per group). * $P < .05$. *i.n.*, Intranasal; *i.p.*, intraperitoneal.

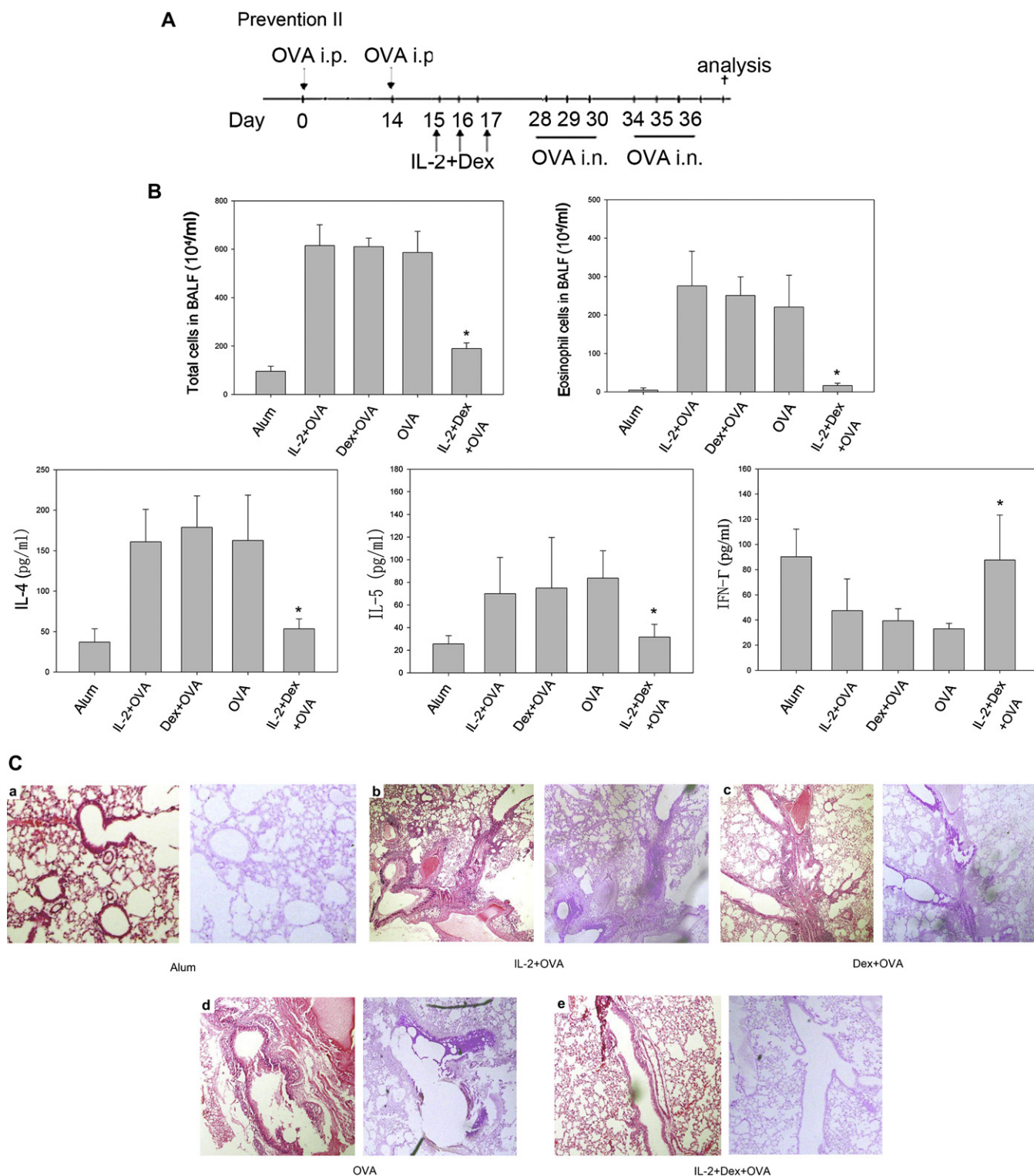


FIG E3. Manifestations of allergic airway disease after IL-2 and dexamethasone (*Dex*) were administered after OVA priming. **A**, IL-2 and dexamethasone were administered for 3 days after OVA priming. **B**, Analysis of lymphocyte cell counts, cytokines in BALF, and lung inflammation after OVA challenge. **C**, Images of lung sections ($\times 40$ magnification). *Left*, H&E staining; *right*, PAS staining. Data are presented as means $\pm$ SDs ($n = 6$ per group). $*P < .05$. *i.n.*, Intranasal; *i.p.*, intraperitoneal.

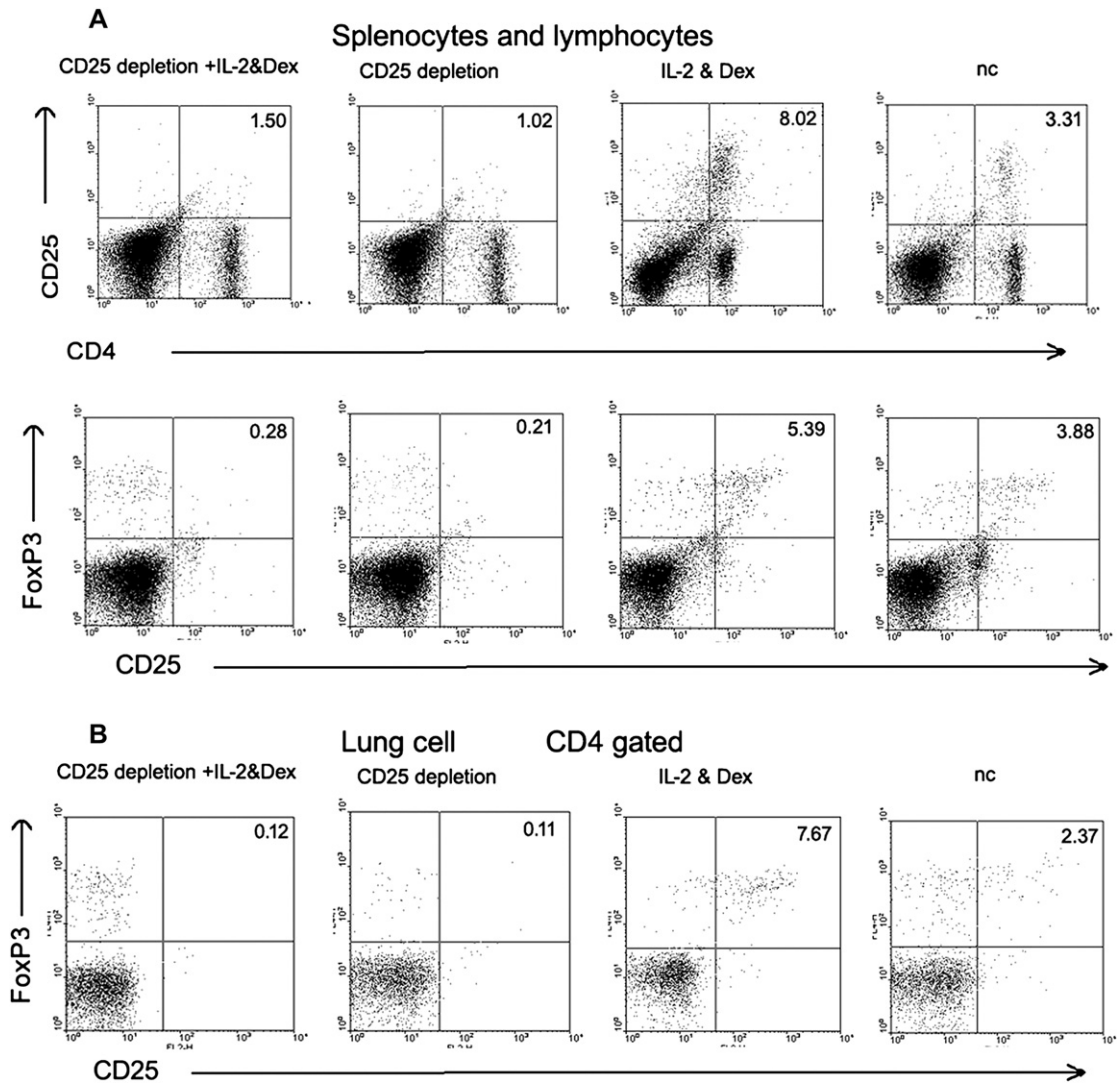


FIG E4. Treg cell analysis after anti-CD25 treatment. Detection of Treg cells after administration of anti-CD25 antibody or other intervention in lymphocytes (**A**) and lung cells (**B**). *Dex*, Dexamethasone.

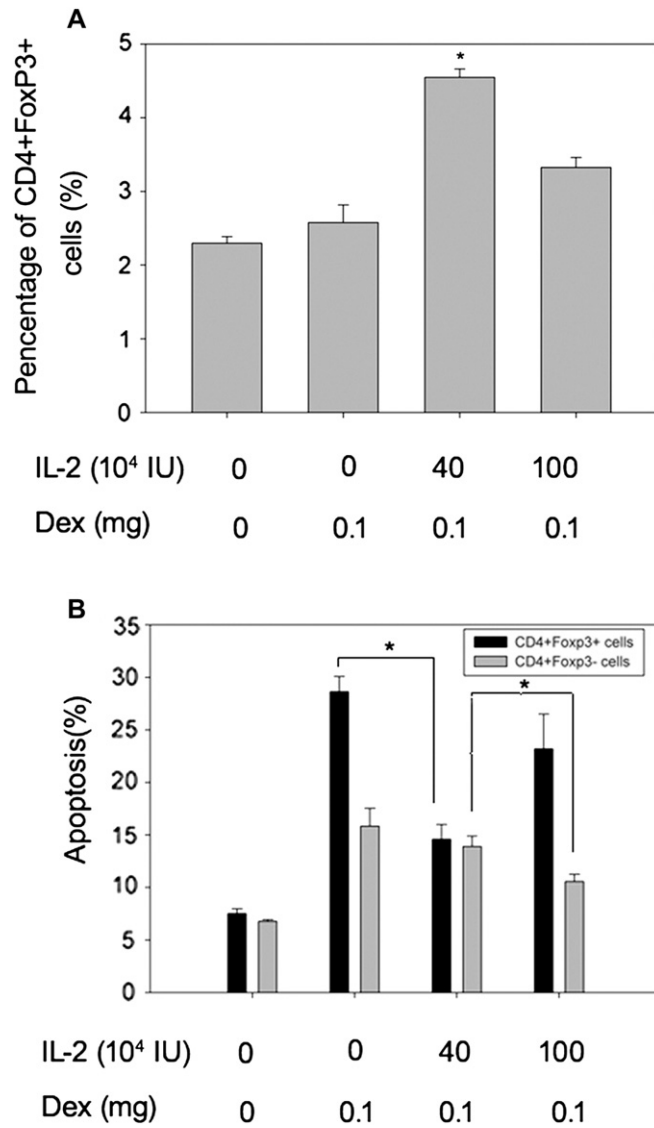


FIG E5. The levels of apoptotic Treg cells and CD4⁺FoxP3⁻ cells after different drug intervention *in vivo*. **A**, The proportion of Treg cells after administration of PBS, dexamethasone (*Dex*) only, 400,000 IU of IL-2 and dexamethasone, or 1,000,000 of IU IL-2 and dexamethasone. **B**, The levels of apoptotic Treg cells and CD4⁺FoxP3⁻ cells were detected after corresponding drug intervention. Data are presented as means $\pm$ SDs ($n = 4$ mice per group). * $P < .05$.

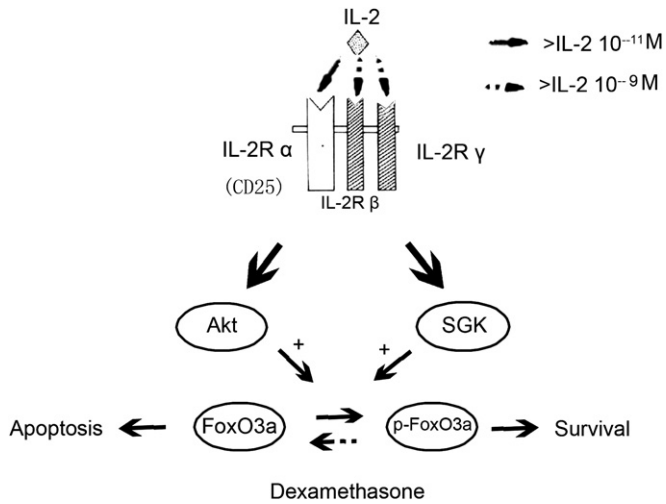


FIG E6. The potential mechanism of IL-2 and dexamethasone-induced upregulation of Treg cells. When the concentration of IL-2 reaches 10^{-11} mol/L, IL-2 can bind CD25, followed by activation of the Akt and SGK pathway, but only Treg cells (CD25 expression) are protected from dexamethasone-induced apoptosis. However, when the concentration of IL-2 reaches 10^{-9} mol/L, IL-2 can bind the IL-2 receptor (*IL-2R*) β and γ chains, resulting in protection of both Treg and effector T cells.