

IL-2 Receptor β -Chain Signaling Controls Immunosuppressive CD4⁺ T Cells in the Draining Lymph Nodes and Lung during Allergic Airway Inflammation In Vivo

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IL-2 influences both survival and differentiation of CD4⁺ T effector and regulatory T cells. We studied the effect of i.n. administration of Abs against the α - and the β -chains of the IL-2R in a murine model of allergic asthma. Blockade of the β - but not the α -chain of the IL-2R after allergen challenge led to a significant reduction of airway hyperresponsiveness. Although both treatments led to reduction of lung inflammation, IL-2 signaling, STAT-5 phosphorylation, and Th2-type cytokine production (IL-4 and IL-5) by lung T cells, IL-13 production and CD4⁺ T cell survival were solely inhibited by the blockade of the IL-2R β -chain. Moreover, local blockade of the common IL-2R/IL-15R β -chain reduced NK cell number and IL-2 production by lung CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells while inducing IL-10- and TGF- β -producing CD4⁺ T cells in the lung. This cytokine milieu was associated with reduced CD4⁺ T cell proliferation in the draining lymph nodes. Thus, local blockade of the β -chain of the IL-2R restored an immunosuppressive cytokine milieu in the lung that ameliorated both inflammation and airway hyperresponsiveness in experimental allergic asthma. These findings provide novel insights into the functional role of IL-2 signaling in experimental asthma and suggest that blockade of the IL-2R β -chain might be useful for therapy of allergic asthma in humans. *The Journal of Immunology*, 2008, 181: 1917–1926.

In asthmatic patients, the reduction of allergic symptoms after successful allergen immunotherapy has been associated with the appearance of IL-10-producing regulatory T cells (T regs)³ (1, 2). IL-2 is an immunoregulatory cytokine which acts as a survival and differentiation factor for CD4⁺ naive T cells as well as for T regs (3). IL-2 signaling requires the dimerization of the IL-2R β - and common γ - (γ_c) chains. Mutations of γ -chain can result in X-linked severe combined immunodeficiency. IL-2, IL-4, IL-7, and IL-9 (whose receptors contain the γ -chain) induce the tyrosine phosphorylation and activation of the Janus family tyrosine kinases, Jak1 and Jak3. Jak1 and Jak3 may then associate with the IL-2R β - and γ -chains (4).

IL-2 binds to the α -chain of the IL-2R, which has been often used as a marker for both T regs and effector T cells (CD4⁺CD25⁺ T cells). By contrast, the β -chain is important for signal transduc-

tion and acts as a common chain for IL-2R and IL-15R (5–10). IL-2 is also involved in the priming of T cells to undergo proliferation and apoptosis. In contrast, IL-15 is essential for cell survival and maintenance of long-lived memory cells and NK cells (11–17). IL-2 elicits cellular actions by binding to the α -chain of its receptor (5–10). This event is followed by recruitment of the β - and the γ -chain molecules into trimeric structures, thereby forming the active IL-2R complex. Binding of IL-2 to the active IL-2R leads to intracellular activation of JAK, which in turn phosphorylates STAT-5 followed by activation of IL-2 target genes. Blockade of the α -chain and, even more effectively, blockade of the β -chain of the IL-2R inhibits STAT-5 phosphorylation and subsequently IL-2 target gene activation. The functional importance of IL-2 signaling in T lymphocytes has been underlined by the finding that such signaling augments T cell activation and Th 2 cell polarization in vitro and in vivo. In this study, we analyzed the role of the two distinct IL-2R chains in a murine model of asthma.

Materials and Methods

OVA sensitization and challenge and anti-IL-2R Ab and IL-2 treatment

Female BALB/cJ mice (6–8 wk of age) received i.p. injections of 100 μ g of OVA (Calbiochem), complexed with alum (Sigma-Aldrich), on days 0 and 14, as previously described (18–20). On days 25, 26, and 27, mice received intranasally (i.n.) 50 or 100 μ g of anti-IL-2R α -chain Ab (purified rat anti-mouse CD25 clone: PC61; BD Biosciences/BD Pharmingen), anti-IL-2R β -chain Ab (purified rat anti-mouse CD122 mAb clone: TM- β 1, K; BD Biosciences/BD Pharmingen), monoclonal anti-mouse IL-13 Ab (R&D Systems), or control Ig (purified rat IgG, Sigma-Aldrich; purified rat IgG2b, K monoclonal Ig isotype standard, BD Biosciences/BD Pharmingen; and purified rat IgG1, λ monoclonal Ig isotype standard, BD Biosciences/BD Pharmingen). The Ab was administered i.n. 30 min before the i.n. OVA challenge. IL-2 (1 or 5 μ g) was injected i.p. at day 0 before the first sensitization. rIL-2 was obtained using the hybridoma cell line X 63/0-mrIL-2 K22 cultured in RPMI 1640 medium containing 5% FBS, 1% glutamine, and 1% sodium

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Received for publication March 21, 2008. Accepted for publication May 26, 2008.

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³Abbreviations used in this paper: T reg, regulatory T cell; i.n., intranasal(ly); AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BALF, BAL fluid.

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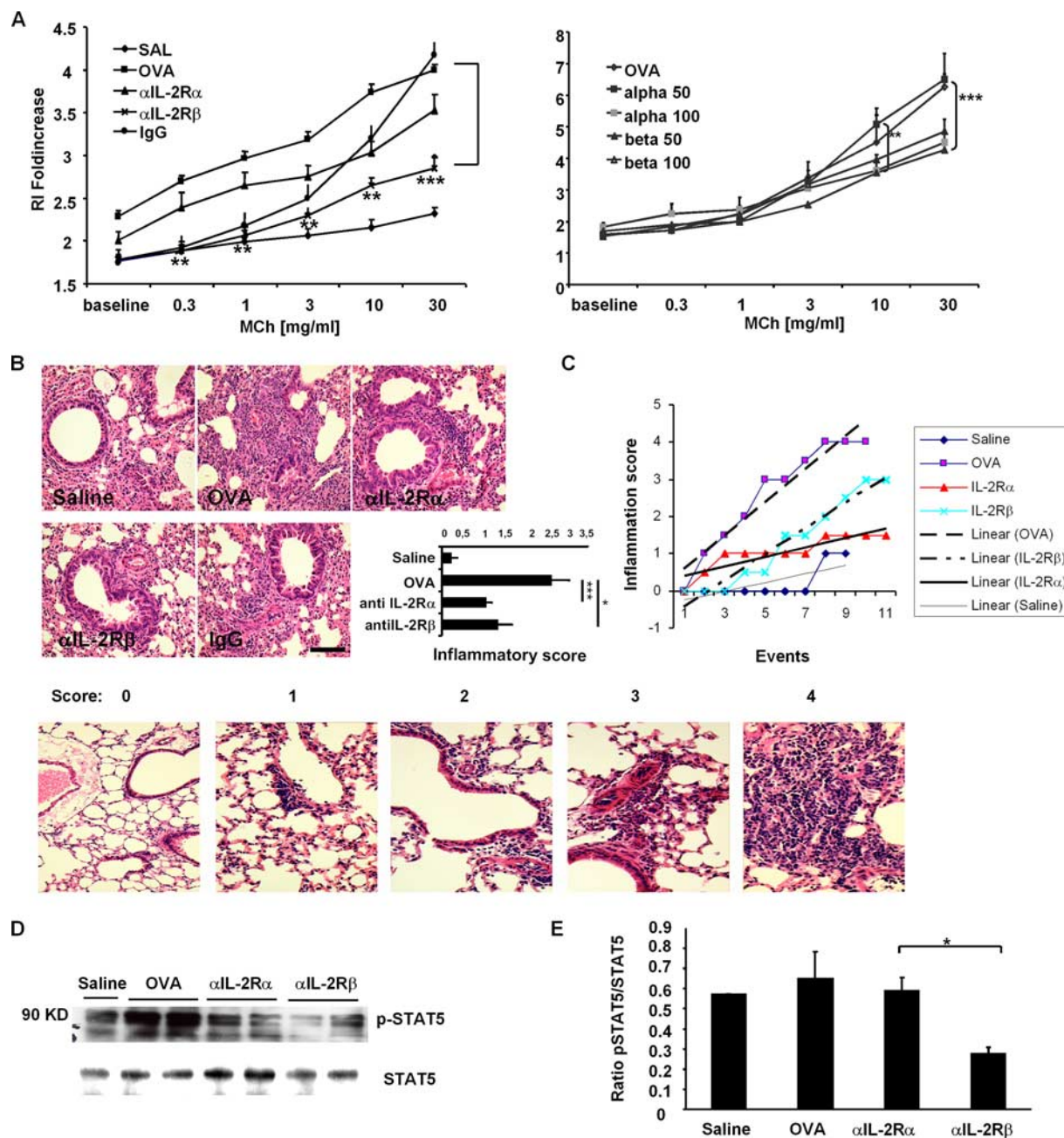


FIGURE 1. Anti-IL-2R β -chain Abs ameliorated AHR and inflammation in a murine model of asthma. *A, Left*, Effects of Ab treatment on AHR in experimental asthma upon OVA sensitization and challenge. One representative experiment of five in OVA-induced AHR and lung inflammation is shown in which four BALB/cJ mice per group were used. Anti-IL-2R β -chain Ab treatment significantly ameliorated AHR (0 mg/ml methacholine (MCh): $p = 0.040$; 0.3 mg/ml MCh: $p = 0.0041$; 1 mg/ml MCh: $p = 0.0028$; 3 mg/ml MCh: $p = 0.0012$; 10 mg/ml MCh: $p = 0.0014$; 30 mg/ml MCh: $p = 9.02E-05$), while anti-IL-2R α -chain Ab treatment had no significant effects ($n = 6$). *Right*, Higher doses (100 μ g/day) of anti-CD25 Abs decreased the AHR as compared with 50 μ g/day. However, these changes were not statistically significant ($p = 0.0664$). Shown is the statistical significance between 50 μ g of anti-CD25 and 50 μ g of anti-CD122 Abs after 10 mg/ml MCh (**, $p = 0.0198$) and after 30 mg/ml MCh (***, $p = 0.00183$). One hundred micrograms per day of anti-CD122 Abs did not ameliorate further the AHR as compared with 50 μ g of anti-CD122 Abs/day ($n = 3$; $p = 0.0336$, 100 μ g/day vs 50 μ g/day anti-CD25 Abs). *B*, Histological assessment of lung inflammation on H&E-stained sections from all five groups. Decreased lung inflammation after anti-IL-2R β -chain Ab treatment was noted. *C*, Pathological score of inflammation in experimental asthma. Scores of one representative experiment are shown ($n = 4$ /group). Scoring was performed as specified in *Materials and Methods* and as shown in the *lower panels*. Histological examples of inflammatory scores 0–4 are shown. Anti-IL-2R Ab treatment significantly suppressed lung inflammation (***, $p < 0.01$; *, $p < 0.05$). *D*, Immunoprecipitation of STAT-5 and STAT-5 phosphorylation analysis (p-STAT-5) in experimental asthma. *E*, Quantification of the p-STAT-5:STAT-5 ratio in the lung in response to IL-2R blockade. Blockade of the IL-2R β -chain led to a down-regulation of STAT-5 phosphorylation. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

pyruvate. The supernatant was collected and concentrated, before extraction of the rIL-2 protein via affinity columns (1 IU = 10 ng of IL-2). The doses of IL-2 given by i.p. injection were between 1 and 5 μ g/20

g of body weight and thus comparable to those used in clinical trials. All experiments were undertaken with an approved license (no. 177-07-991-32, obtained from the Ethical Review Board, Rheinland-Pfalz).

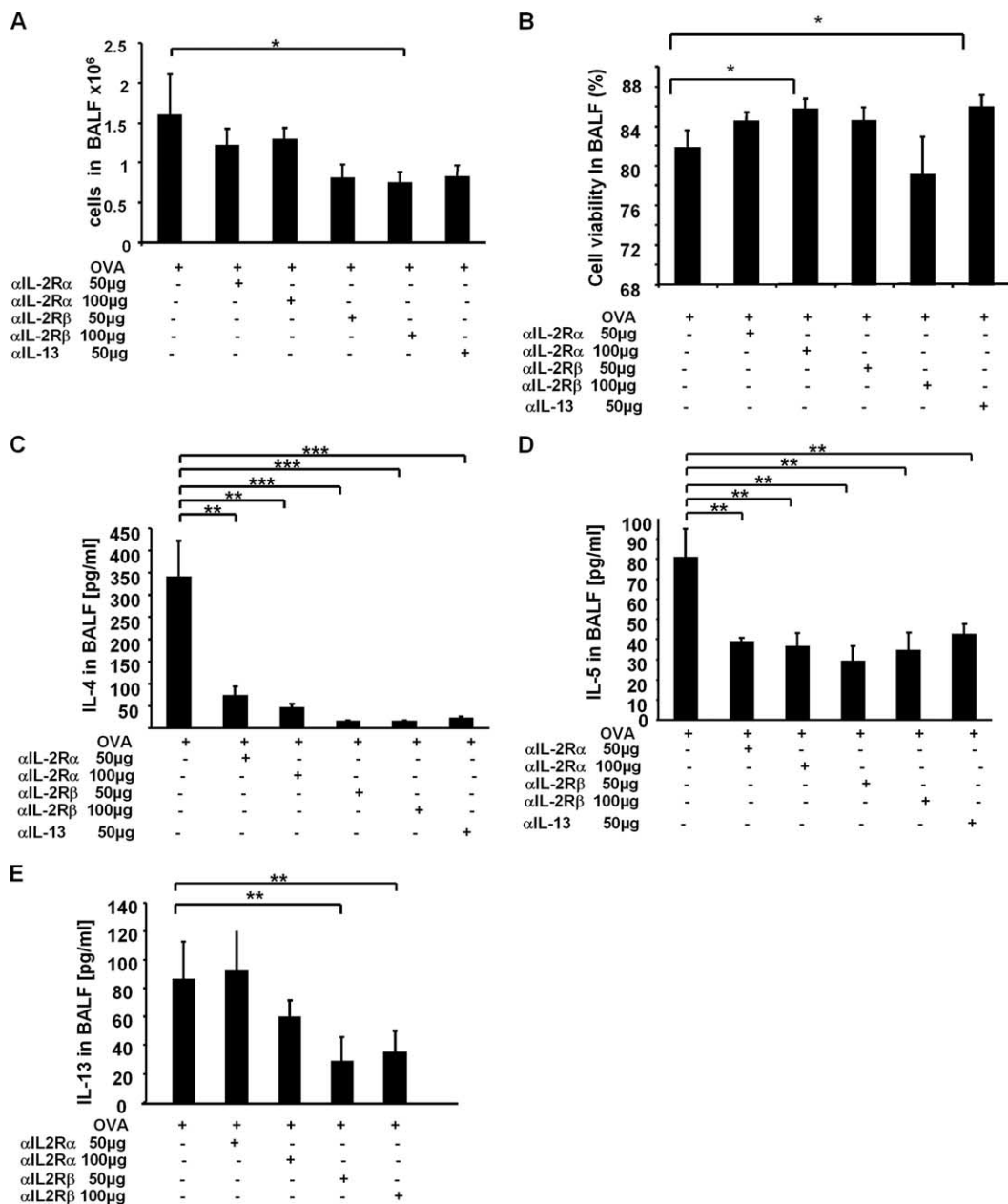


FIGURE 2. Blockade of the α - and β -chains of the IL-2R differentially down-regulated Th2-type cytokines in the airways in a murine model of asthma. **A**, Analysis of cells in BALF of mice after anti-IL-2R and -IL-13 Ab therapy (50 μ g of Ab/day i.n.) in experimental asthma. A significantly decreased number of cells was noted in the airways after anti-IL-2R β -chain Ab treatment ($p = 0.047$; $n = 4-6$). **B**, Higher doses of anti-CD25 Abs (100 μ g/day) induced the cell viability in the BALF in a murine model of asthma (*, $p < 0.05$; $n = 6$ /group). **C-E**, Cytokine levels in BALF: anti-IL-2R α -chain as well as anti-IL-2R β -chain Ab-treated mice showed a significant reduction in IL-4 (**C**: $p = 0.029$, $p = 0.0027$, $p = 0.00061$, $p = 0.00062$, $p = 0.00072$; $n = 4-6$) and IL-5 (**D**: $p = 0.0036$, $p = 0.010$, $p = 0.0092$, $p = 0.0086$, $p = 0.010$) levels. By contrast, IL-13 levels (**E**; $n = 4-6$) were specifically decreased in the airways of mice treated with anti-IL-2R β -chain Abs after OVA sensitization and challenge. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Assessment of airway reactivity by invasive body plethysmography

Airway reactivity was assessed in six mice per group. Three to four independent experiments were performed. Airway hyperresponsiveness (AHR) in mice was evaluated at day 28, by using an invasive, whole-body plethysmograph, registering the responses to varying doses of methacholine as previously described (20).

Lung histology

Twenty-four hours after the last i.n. challenge with either OVA or saline, lungs were analyzed by histology (day 28). The right lung was frozen immediately and stored in liquid nitrogen until use. The left lung was fixed

in 10% formalin, dehydrated, mounted in paraffin, sectioned, and stained with H&E. Lungs were analyzed by the same pathologist (H. A. Lehr).

For quantification of perivascular inflammation severity, digital high-power ($\times 400$) images were taken by a pathologist blinded to the group assignment of the H&E sections from three random peribronchovascular vessels per lung using a standard diagnostic microscope (BX45; Olympus) and a digital camera (C5050; Olympus). Images were imported into Photoshop (version 7; Adobe Systems) run on a Macintosh computer (G5; Apple). Through the use of duplicated layers and the rubber stamp technique, a rim of exactly 300- μ m width was selected around each vessel. The adjacent bronchus was manually excluded from analysis by covering the respective area with a white stamp. Then, the number of cells was manually counted on black and white printouts and expressed as cells per

millimeter-squared (the analyzed surface area being assessed using the histogram tool in Photoshop). Complete chromogen separation and analysis in double immunohistochemical stainings was performed using Photoshop-based image analysis. The cellular density in control lungs, representing cells of the normal alveolar walls and the fine interstitium, was averaged and this value was subtracted from the data obtained in lungs from all treated animals (normal alveolar walls plus interstitium plus inflammatory cells).

The inflammation of the lungs was further assessed by a pathologist blinded to the experimental group assignments of the individual lungs. Inflammation was graded using a four-tier score where: 0, no inflammation; 1, rare occasional inflammatory cells around isolated peribronchial blood vessels; 2, accumulations of scant inflammatory cells around peribronchial vessels in more than one site; 3, multifocal inflammation around peribronchial vessels, easily visible at $\times 4$ magnification; and 4, severe peribronchial inflammatory infiltration at multiples sites. Some representative images are reported on the *lower panels* in Fig. 1B. Results are reported for each individual observation that was performed twice with the best fitting line for the different observation of each group (see Fig. 1B, *lower panels*). The results are also summarized in a graph (Fig. 1B) as mean values $\pm$ SEM ($n = 8-11$).

Collection and analysis of bronchoalveolar lavage (BAL) fluid (BALF) and cytopins

Twenty-four hours after the last i.n. challenge with either OVA or saline at day 27, BAL of the right lung was performed after tying off the left lung at the main stem bronchus. Total BALF (1 ml of saline three times) was collected and cells were counted using a 100- μ l aliquot. Samples were centrifuged at 1200 rpm for 5 min at 4°C, and cell pellets were resuspended in PBS. Cell viability in BALF was analyzed by the vital exclusion dye trypan blue (0.4%; Invitrogen). Supernatants were analyzed by specific ELISA using commercially available kit systems as described below.

Flow cytometric analysis and intracellular staining

To routinely ascertain the purity (purity >94%) of the CD4⁺ T cell isolation, 5×10^5 cells were washed with 1 ml of PBS and incubated for 30 min in 100 μ l of PBS containing 5 μ g/ml anti-CD4 Abs (L3T4-PE; BD Pharmingen). The total cells of the lung and spleen were stained with FACS Abs for 30 min at 4°C, washed in PBS, and then analyzed with a FACSCalibur (BD Pharmingen). The following FITC-, PE-, allophycocyanin-, or PE-Cy5.5-conjugated mAbs were used for flow cytometry: anti-CD122 (TM- β 1), anti-CD4 (L3T4), anti-CD25 (3C7), and (BD Pharmingen and eBioscience). For the intracellular detection of Foxp3⁺, T cells and whole cell suspensions of the draining lymph nodes were washed and incubated with Abs directed against CD4 (L3T4; BD Pharmingen) and CD25 (7D4 or PC61; eBioscience) for 30 min at 4°C, fixed, and permeabilized for 45 min at 4°C and finally incubated with anti-Foxp3 Abs for 30 min at 4°C (FJK-16s; eBioscience). All Abs and their isotype controls were purchased from BD Pharmingen.

For intracellular staining of IL-13, freshly isolated lung cells were stimulated with 1 ng/ml PMA (Sigma-Aldrich), 1 μ M ionomycin (Sigma-Aldrich), and GolgiStop (BD Pharmingen) for 4 h. Cells were stained with anti-CD4 (L3T4; BD Pharmingen), and anti-CD49b (DX5; BD Pharmingen) Abs for 30 min at 4°C. Thereafter, cells were fixed with fixation/permeabilization solution (eBioscience) for 45 min at 4°C and washed with permeabilization buffer (eBioscience). Cells were then incubated with PE-labeled Abs against IL-13 (eBio13A; eBioscience) for 30 min at 4°C in permeabilization buffer and washed once. The intracellular Foxp3 (FJK-16a; eBioscience) staining was performed using the same procedure without cell stimulation.

Functional studies on CD4⁺ T cells purified from the lung and draining lymph nodes

Lungs were removed from mice; lung CD4⁺, CD4⁺CD25⁻, and CD4⁺CD25⁺ T cells were isolated as previously described (18, 21). To detect apoptotic cells, CD4⁺ T cells were treated as previously described for TUNEL assays (22). Lung CD4⁺ T cells, cultured in a 24-well plates with anti-CD3 Abs, were fixed with freshly prepared 4% paraformaldehyde in PBS (pH 7.4) and washed twice with PBS. The cells were treated with a 0.1% Triton X-100/0.1% sodium citrate permeabilization solution and washed three times with PBS. Fifty microliters of the TUNEL (TdT-dependent dUTP-biotin nick-end labeling) reaction solution (In Situ Cell Death Detection kit, Fluorescein; Roche Molecular Biochemicals) was added to the sample and incubated for 60 min in a humidified atmosphere-chamber at 37°C in the dark. Slides were rinsed three times with PBS and analyzed by a fluorescence microscope (Olympus Optical).

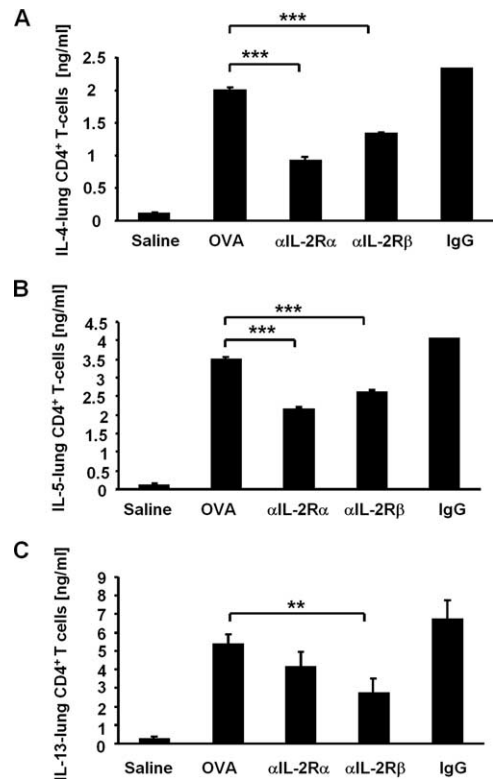


FIGURE 3. In vivo blockade of the α - and β -chains of the IL-2R differentially regulated Th2 cytokine production in the lung in a murine model of asthma. Lung CD4⁺ T cells were isolated from the different groups of mice and cultured overnight with RPMI 1640 medium/5% FBS and challenged by plate-coated anti-CD3 Abs. The day after supernatants were collected and stored at -20°C , cytokine production was assessed in T cell supernatants by ELISA. CD4⁺ T cells from lungs of anti-IL-2R α -chain and anti-IL-2R β -chain Ab-treated mice showed a significant reduction in IL-4 (A) (anti-IL-2R α -chain: $p = 1.54\text{E-}06$, anti-IL-2R β -chain: $p = 0.00015$; $n = 4$) and IL-5 (B) (anti-IL-2R α -chain: $p = 1.6\text{E-}06$, anti-IL-2R β -chain: $p = 6.7\text{E-}06$; $n = 4$) production as compared with control T cells from OVA-treated mice. However, IL-13 (C) ($p = 0.011$; $n = 8$) production by lung T cells was significantly suppressed after treatment with anti-IL-2R β -chain Abs only. The number of CD4⁺ T cells did not change in the lung after Ab treatment (data not shown).

In three independent experiments, CD4⁺ draining lymph node T cells were directly purified from isolated cell suspension by incubation at 4°C for 15 min with 10 μ l of anti-mouse CD4 L3T4 microbeads per 10^7 total cells. Cells were positively sorted in a multiparameter magnetic cell sorter system (MACS; Miltenyi Biotec). A total of 10^5 cells/well CFSE-labeled (Molecular Probes) CD4⁺ T cells (10^5 cells/well) were then CFSE-labeled (Molecular Probes/Invitrogen) and coincubated in the presence of APCs (A20 a murine B lymphoma cell line; 10^4 cells/well) in the presence of soluble anti-CD3 Abs (2.5 μ g/ml) overnight. To avoid proliferation of the A20 murine B lymphoma cell line in vitro, we pretreated this cell line with 60 μ g/ml mitomycin C at 37°C for 30 min. After 20 h, the starting level of incorporated fluorescence was recorded (M1). The proliferation of the CD4⁺(CFSE⁺) T cells was determined 4 days later and was expressed as the percentage of daughter cells that would reach generation M5 or M6 (mitoses 5–6). Two to three pools with two lungs per pool for each experimental group were analyzed.

ELISA

Mouse IL-2 (3.1–200 pg/ml), IL-4 (7.48–500 pg/ml), IL-5 (15.6–1000 pg/ml), and IL-10 (31.25–2000 pg/ml) were detected using a specific sandwich ELISA (OptEIA; BD Biosciences/BD Pharmingen). Murine IL-13 (39.2–2500 pg/ml) was detected using an ELISA kit (R&D Systems). TGF- β (31.25–2000 pg/ml) analysis was performed by using purified rat anti-mouse, -human, and -pig TGF- β 1 as capture Abs (BD Biosciences/BD Pharmingen) and biotinylated rat anti-mouse, -human, and -pig TGF- β 1

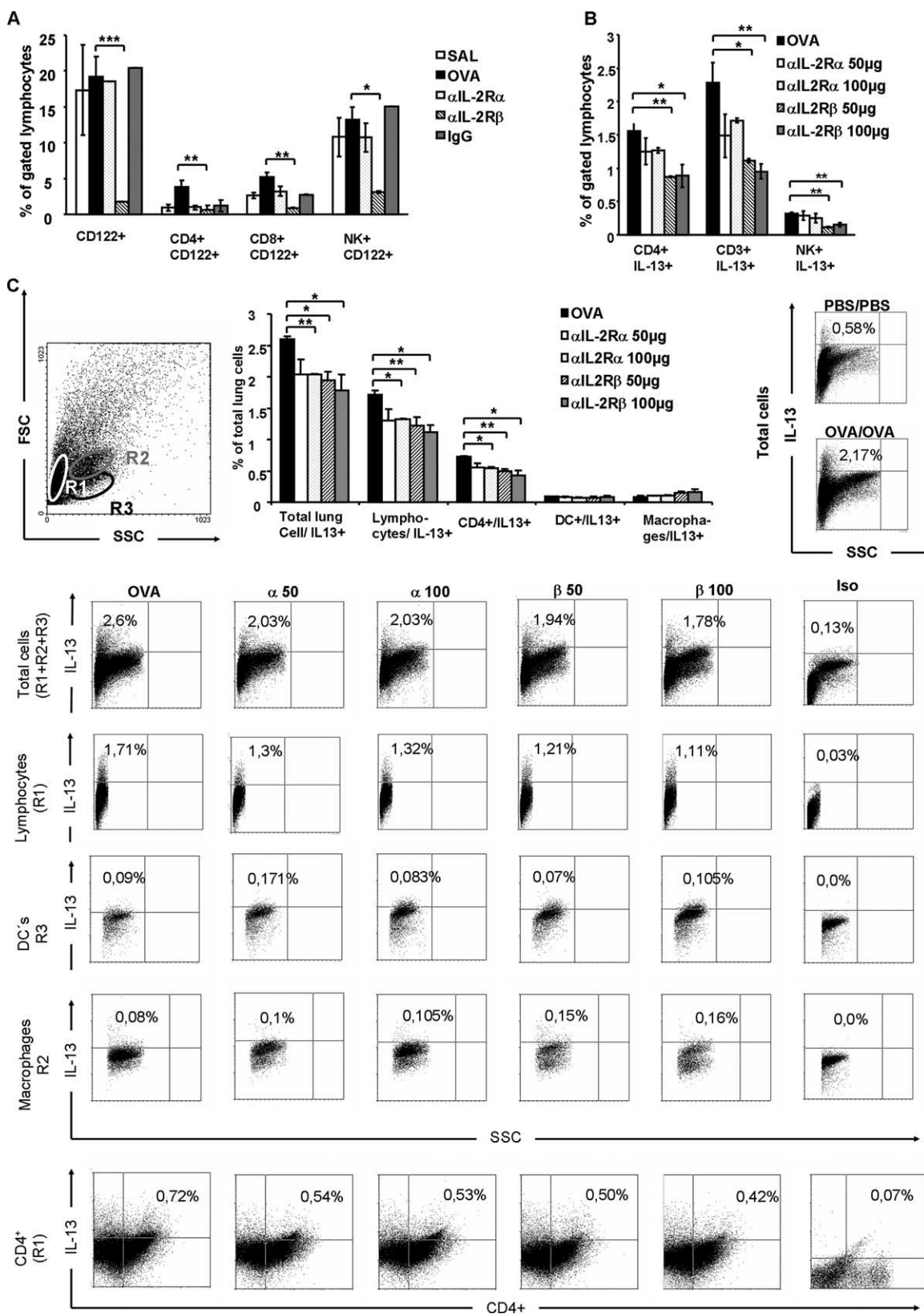


FIGURE 4. CD4⁺ T cells are the major source of IL-13 production in the lung in experimental asthma. Lung cells expressing the β -chain of the IL-2/IL-15R (CD122) were analyzed by FACS after sensitization and challenge of wild-type mice with OVA. **A**, Notably, NK cells (NK) represented the majority of cells in the lung expressing the β receptor chain, but in these cells OVA Ag challenge did not influence CD122 expression as compared with the saline control group. The blockade of the β -chain led to a significant down-regulation of the number of CD4⁺CD122⁺ T cells, CD8⁺CD122⁺ T cells, and NK⁺CD122⁺ cells in the lung ($p = 0.035$ for CD4; $p = 0.012$; and $p = 0.0017$ for CD8, and $p = 0.015$ for NK cells, respectively). **B**, Intracellular staining for IL-13 was performed followed by cell surface staining for CD4, CD3, and NK markers. The percentage of CD4⁺IL-13⁺ ($p = 0.013$, $p = 0.031$), CD8⁺IL-13⁺ ($p = 0.030$, $p = 0.0076$), and NK⁺IL-13⁺ T cells ($p = 0.016$, $p = 0.0092$) was down-regulated after different doses of anti-CD122 Ab therapy, as shown by gating on the lymphocyte area ($n = 3$). **C**, Detailed FACS analysis of this staining by using three gates: R1, gate on lymphocytes; R2, gate

(BD Biosciences/BD Pharmingen) polyclonal Abs. rTGF- β was purchased from R&D Systems. To activate TGF- β , 200- μ l samples were pretreated with 10 μ l of 1 N HCl for 30 min at 37°C. Samples were then neutralized by adding 10 μ l of 1 N NaOH as previously described (19).

Protein extraction, Western blot, and immunoprecipitation analysis

Tissue proteins were extracted and immunoprecipitation and Western blots were performed as previously described using anti-STAT-5 and -p-STAT-5, respectively (eBioscience) (22). Briefly, tissue was homogenized in PBS and protein was extracted in the presence of protease inhibitors (6.75% aprotinin, 312 μ g/ml trypsin inhibitor) and detergent (Nonidet). Protein concentrations were determined by spectrophotometry with the Bio-Rad protein assay.

For immunoprecipitation, 250 μ g of total lung proteins was precleared with 1 μ g of appropriate IgG according to the primary Ab and 20 μ l of A/G plus agarose (Santa Cruz Biotechnology) for 30 min at 4°C. After centrifugation at 2500 rpm for 5 min, supernatant was collected and incubated with 2 μ g of primary Ab (STAT-5) for 1 h at 4°C followed by addition of 20 μ l of A/G plus agarose. The immunoprecipitation was completed by incubation at 4°C overnight under rotating conditions. The next day, the pellet was washed three times with PBS and resuspended in 50 μ l of PBS. Western blots were performed as described above with one-third of immunoprecipitated protein, blotted in a nitrocellulose membrane, and incubated with anti-phosphotyrosine residues of STAT-5 (Santa Cruz Biotechnology). Specific binding was visualized with the ECL Western Blotting Detection System (Amersham Biosciences) according to the manufacturer's instructions after a 1-h incubation with the corresponding secondary, HRP-conjugated Ab (1/2000 dilution in blocking solution; Amersham Biosciences).

Statistical analysis

Differences were evaluated for significance ($p < 0.05$) by the Student two-tailed t test for independent events (Microsoft Excel, version 2000).

Results

Blockade of the β -chain of the IL-2R ameliorates both AHR and inflammation in a murine model of asthma

To assess the effects of IL-2 signaling in experimental asthma under in vivo conditions, we took advantage of neutralizing Abs against the IL-2R α - (CD25) and β - (CD122) chains in an experimental model of asthma induced by OVA sensitization and challenge. We observed a reduction of OVA induced AHR after anti-IL-2R β -chain Ab treatment (Fig. 1A, *left panel*). By contrast, blockade of the IL-2R α -chain had no significant effects on AHR in this model at low doses (50 μ g/day; Fig. 1A, *right panel*). Higher doses of anti-CD25 Abs (100 μ g/day) suppressed the increased AHR, without reaching statistical significance (Fig. 1A, *right panel*). The inflammatory cell infiltrate around the bronchi and vessels, induced by OVA, was reduced after i.n. application of anti-IL-2R α -chain as well as anti-IL-2R β -chain Abs (50 μ g/day, respectively; Fig. 1, B and C). No influence on total IgE and number of lung CD4⁺ T cells was observed in the different groups (data not shown). Moreover, a significant increase in the cell viability in the BALF was observed after higher doses of anti-IL-2R α -chain Abs (100 μ g/day) and after 50 μ g of anti-IL-13 Abs per day (Fig. 2B).

The blockade of IL-2R signaling in experimental asthma led to down-regulation of STAT-5 phosphorylation (23) especially after blockade of the IL-2R β -chain (Fig. 1, D and E) demonstrating successful targeting of the intracellular IL-2-signaling pathway in the lung upon i.n. Ab application.

Blockade of the α - and β -chains of the IL-2R differentially regulates Th2 cytokine production and CD4⁺ T cell apoptosis in the lung in a murine model of asthma

We subsequently determined cytokine production in the airways (Fig. 2) and by purified lung T cells (Fig. 3) upon Ab therapy using different Ab doses. It was found that anti-IL-2R β Ab treatment (50–100 μ g/day) led to significant suppression of Th2 cytokine levels (IL-4, IL-5, and IL-13) in the airways (Fig. 2, C–E, respectively) and Th2 cytokine production by lung T cells (Fig. 3, A–C, respectively), as compared with untreated and IgG treated control mice. These effects in the BALF were comparable to anti-IL-13 Ab treatment. However, blockade of the IL-2R α -chain caused significantly decreased production of IL-4 and IL-5 without affecting IL-13 production in the airways and by lung CD4⁺ T cells after OVA challenge (Figs. 2 and 3, respectively). These results indicate that blockade of the IL-2R α -chain controls, specifically, IL-4 and IL-5 production by lung CD4⁺ T cells, whereas blockade of the IL-2R β -chain also inhibited production of IL-13 (19, 20, 24).

CD122 is expressed predominantly on NK cells in the lung, but NK⁺CD122⁺ cells are not induced after allergen challenge in a murine model of allergic asthma

To further explore the cells expressing the β -chain of the IL-2R, we next analyzed lung cells in our model of allergic airway inflammation and hyperresponsiveness by FACS analysis. As shown in Fig. 4A, it was found that 20% of the gated lymphocytes express this receptor component. Notably NK cells (NK) represented the majority of cells in the lung expressing this receptor chain. In contrast to CD4⁺ and CD8⁺ T cells, the β -chain of the IL-2R was not up-regulated by allergen challenge on NK cells in experimental asthma. However, blockade of the IL-2R β -chain led to a significant down-regulation of the number of NK⁺CD122⁺, CD4⁺CD122⁺, and CD8⁺CD122⁺ T cells. Consistently, the total number of CD122⁺ T cells was reduced upon Ab treatment.

CD4⁺ T cells are the major source of IL-13 in the lung in this experimental model of asthma

We next asked whether CD4⁺ T cells would be the major source of IL-13 in the lung in this murine model of asthma, as other cells are known to produce this cytokine. To address this point, we performed intracellular staining for IL-13 followed by cell surface staining for CD4, CD3, and NK markers to identify different cell types producing IL-13 in the lung in our murine model of asthma by FACS analysis before and after the blockade with different Abs (Fig. 4, B and C).

As shown in Fig. 4C, ~2.7% of the cells in the lung expressed IL-13 intracellularly. Of these cells ~1.7% were identified as lymphocytes after gating on R1. Moreover, we set a second gate, called R2, on big autofluorescent cells containing macrophages and mast cells and a third gate called R3 on dendritic cells. As shown in Fig. 4C, very few IL-13-positive cells were found in gates R2 and R3, whereas almost all IL-13-positive cells were detected in the lymphocyte gate.

Moreover, as shown in Fig. 4B, when gated on lymphocytes, CD4⁺/IL-13 T cells were the predominant cell population bearing the TCR (CD3⁺). By contrast, NK/IL-13⁺ T cells represented <20% of the CD3/IL-13-producing cells only. We thus concluded

on big autofluorescent cells containing macrophages and mast cells; and R3, gate on dendritic cells. As shown in representative dot plots, very few IL-13-positive cells were found in gates R2 and R3, whereas almost all IL-13-positive cells were detected in the lymphocyte gate (R1) (*, $p = 0.05$, **, $p = 0.01$; $n = 3$). As negative and positive control for IL-13 intracellular staining, stained lung cells from naive mice (PBS/PBS) and OVA-sensitized and -challenged mice (OVA/OVA) were used, respectively (*right panel*).

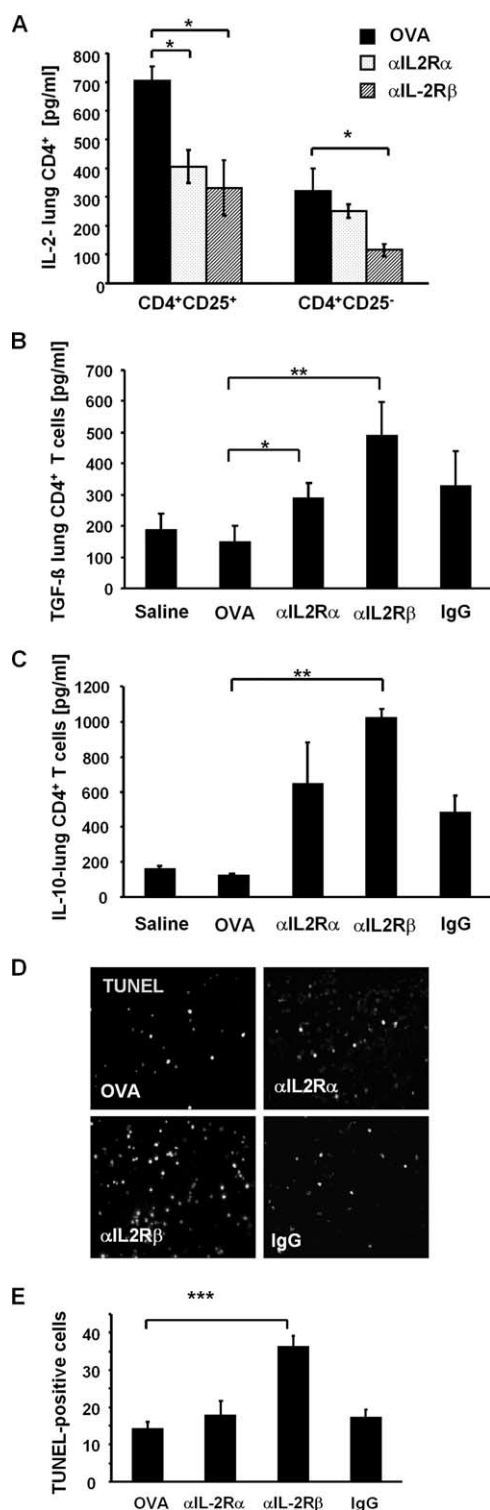


FIGURE 5. Decreased IL-2 and increased TGF- β and IL-10 release by lung CD4⁺ T cells isolated from anti-IL-2R β -chain Ab-treated mice. **A**, IL-2 production by lung T lymphocytes. Lung CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells isolated from the lung of anti-IL-2R β -chain Ab-treated mice secreted significantly less IL-2 as compared with those isolated from untreated or anti-IL-2R α -chain Ab-treated mice ($n = 4$; $p = 0.011$ and $p = 0.46$ for CD4⁺CD25⁺ and $p = 0.012$ for CD4⁺CD25⁻). **B**, Increased levels of TGF- β were produced by lung T cells after in vivo treatment with anti-IL-2R α -chain and anti-IL-2R β -chain Abs ($p = 0.047$ and $p = 0.11$, respectively; $n = 6$). **C**, IL-10 release by lung CD4⁺ T cells was increased after in vivo treatment with anti-IL-2R β -chain (CD122) Abs ($p = 0.0023$; $n = 5-6$). **D**, Increased number of apoptotic (TUNEL positive) lung CD4⁺ T cells induced by local anti- β -chain Ab treatment

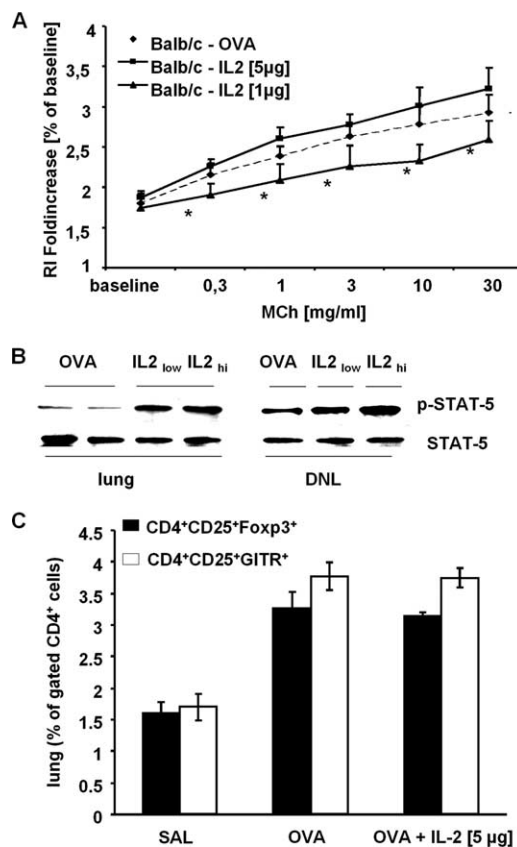


FIGURE 6. Intraperitoneal administration of rIL-2 regulates AHR in mice. **A**, Decreasing the doses of IL-2 significantly decreased AHR (upper panel) in BALB/c mice ($n = 6$). **B**, Increased STAT-5 phosphorylation in the lung and draining lymph nodes after i.p. delivery of IL-2. **C**, IL-2 treatment did not enhance CD4⁺CD25⁺Foxp3⁺ or CD4⁺CD25⁺GITR⁺ T cells in the lungs of treated mice.

that CD4⁺ T cells are the main source of IL-13 in the lung in our model of allergic asthma.

Effect of IL-2 signaling on AHR and lung CD4⁺ T lymphocytes

Effector CD4⁺ T cells as well as CD4⁺ T regs survive in the presence of different doses of IL-2 (25). As shown in Fig. 5A, CD4⁺CD25⁺ T cells produced significantly more IL-2 as compared with the CD4⁺CD25⁻ T cell population after OVA sensitization and challenge. Because T regs are not known to produce IL-2, the IL-2-producing CD4⁺CD25⁺ cells are likely CD4⁺CD25⁺ T effector cells. After blockade of the IL-2R β -chain, CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells showed significantly reduced production of IL-2 (Fig. 5A). We then asked whether an environment with low amounts of IL-2 would favor T reg development and found that lung CD4⁺ T cells isolated from mice treated with anti IL-2R β -chain released increased levels of the immunosuppressive cytokines TGF- β and IL-10 (Fig. 5, B and C, respectively). Thus, low IL-2 production by lung CD4⁺CD25⁻ T cells and increased production of IL-10 and TGF- β by lung CD4⁺ T cells were key characteristics of the local cytokine profile induced by blockade of the IL-2R β -chain in experimental asthma.

We then tested the possibility that the inhibition of IL-2R β signaling would induce apoptosis of activated CD4⁺ T cells after

($p = 4.435 \times 10^{-5}$). Data are reported as number of TUNEL-positive cells per field ($\times 400$ magnification). **E**, Quantification is expressed as number of TUNEL-positive lung CD4⁺ T cells per high-power field.

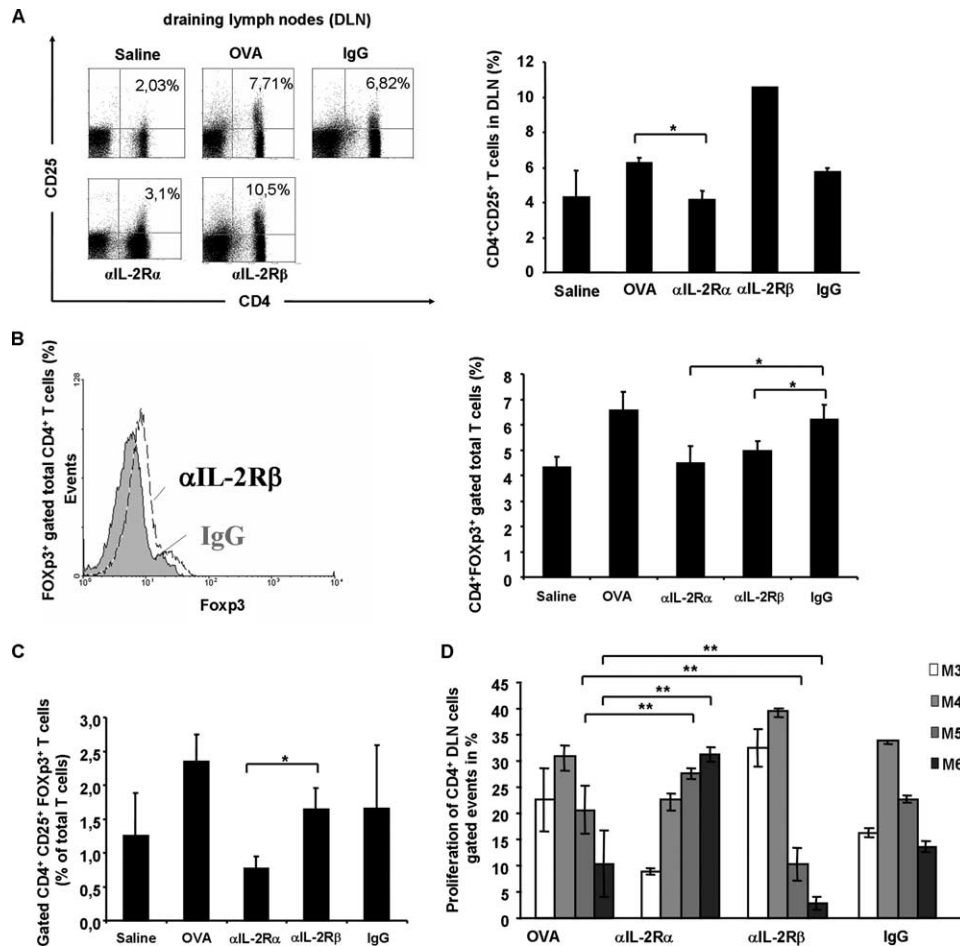


FIGURE 7. Local delivery of Abs to the β - and α -chains of the IL-2R differentially regulates CD4⁺ T cell proliferation in draining lymph nodes in a murine model of asthma. **A**, Analysis of CD4⁺CD25⁺ T cells in draining lymph nodes in experimental asthma. A reduced percentage of CD4⁺CD25⁺ T cells was noted in the draining lymph nodes after in vivo anti-IL-2R α -chain Ab treatment ($n = 3$; $p = 0.021$). **B**, Analysis of Foxp3-expressing regulatory CD4⁺ T cells in the draining lymph nodes. A representative histogram is shown on the left. Both anti-IL-2R α -chain and anti-IL-2R β -chain Abs down-regulated the number of CD4⁺Foxp3⁺ T cells in the draining lymph nodes of treated mice ($n = 3$; $p < 0.05$). **C**, Mice which had been treated in vivo with anti-IL-2R α -chain Abs showed a reduced percentage of CD4⁺CD25⁺Foxp3⁺ T cells as compared with those isolated from mice treated with anti-IL-2R β -chain Abs ($p = 0.029$; $n = 3-4$). **D**, Decreased proliferation of CD4⁺ T cells from draining lymph nodes isolated from mice treated in vivo with the anti IL-2R β -chain Ab as compared with OVA-sensitized and -challenged mice treated in vivo with IgG (M5, $p = 0.00055$; M6, $p = 0.000155$). In this experiment, M3, M4, M5, M6 designate the progressive number of mitoses (M) to estimate how rapidly a cell population proliferates.

IL-2/IL-15 deprivation. Accordingly, TUNEL assays on isolated lung CD4⁺ T cells were performed to detect local apoptotic cells. It was found that the blockade of the IL-2R β -chain leads to a significant increase of CD4⁺ T cell apoptosis in the lung of treated mice (Fig. 5, D and E).

We next investigated the role of IL-2 in asthma by injecting different doses of IL-2 i.p. in OVA sensitized and challenged mice. We found that IL-2 controlled AHR in a dose-dependent manner, where higher doses of IL-2 (5 μ g) induced a significantly higher AHR as compared with lower doses (Fig. 6A). The effects of this treatment were further characterized by augmented STAT-5 phosphorylation in the lung and draining lymph nodes of IL-2-treated mice as compared with controls (Fig. 6B) without changes in CD4⁺CD25⁺Foxp3⁺ T cells (Fig. 6C).

Local delivery of Abs to the β - and α -chains of the IL-2R differentially regulates CD4⁺CD25⁺Foxp3⁺ T regs and CD4⁺ T cell proliferation in draining lymph nodes in a murine model of asthma

In a subsequent series of experiments, we addressed the question of whether the protection seen in experimental asthma after i.n.

delivery of Abs directed against the β -chain of the IL-2R would be the result of expanded or activated T regs in the lung. As CD4⁺CD25⁺ T regs in the periphery specifically express the forkhead family transcription factor Foxp3, we determined Foxp3 expression in CD4⁺CD25⁺ T cells in experimental asthma (26, 27). Interestingly, intracellular FACS analysis showed that i.n. delivery of Abs to the IL-2R α -chain significantly reduced CD4⁺CD25⁺Foxp3⁺ T regs in the local draining lymph nodes (Fig. 7C), as compared with treatment with Abs against the IL-2R β -chain. Furthermore, when gated on total lymphocytes in the draining lymph nodes a significant decrease in CD4⁺Foxp3⁺ T cell number was observed both after blockade of the α - and β -chains of the IL-2R (Fig. 7B). In a final series of experiments, we aimed at characterizing the immunosuppressive properties of CD4⁺ T cells isolated from the draining lymph nodes of mice treated with anti-IL-2R Abs. We found that CD4⁺ T cells isolated from the draining lymph nodes of mice treated with anti-IL-2R β -chain Abs proliferated to a significantly lesser extent as compared with those isolated from untreated and IgG-treated OVA-challenged mice (Fig. 7D). Interestingly, IL-2R α -chain blockade induced increased proliferation of CD4⁺ T cells isolated from the

draining lymph nodes. This effect was consistent with the decrease of CD4⁺CD25⁺ T effector cells in the draining lymph nodes (Fig. 7A) and the blockade of T regs upon administration of the PC61 Ab (27).

Discussion

Previous studies demonstrated an expansion of CD4⁺CD25⁺ T cells accompanied by a reduction of regulatory CD4⁺CD25⁺Foxp3⁺ T cells in the lung of patients with allergic asthma (28, 29). As IL-2 is a key growth factor for both effector and regulatory T lymphocytes (30–32), we addressed here the role of IL-2 signaling in T cells in a murine model of allergic asthma induced by OVA. In these studies, we took advantage of neutralizing Abs against the α - (CD25) and β - (CD122) chains of the IL-2R (5–10). Interestingly, such neutralizing Abs against the α - and β -chains of the IL-2R have already been used in clinical trials for chronic inflammatory diseases such as ulcerative colitis (33) and are currently under investigation in patients with allergic asthma (<http://clinicaltrials.gov/show/NCT00028288>).

We thus asked in this study whether IL-2 signaling plays a role in the effector phase of allergic airway inflammation and whether antagonizing the function of IL-2 can be used for the therapy of allergic airway inflammation and AHR. Furthermore, the potential mechanisms of action of such therapy remained to be elucidated. We found that IL-2 signaling controls the balance between effector T cells and T regs in experimental asthma by means of different receptor components. Specifically, our data suggest that signaling via the IL-2R α -chain supports production of the Th2 cytokines IL-4 and IL-5 in the lung, whereas IL-2R β -chain signaling controls local IL-13 production. In addition, blockade of IL-2R β -chain signaling induced the development and functional activity of immunosuppressive CD4⁺ T in the draining lymph nodes. Different types of CD4⁺ T cells with suppressive function have been recently described including CD4⁺CD25⁺Foxp3⁺ cells (18, 25) which were down-regulated by the blockade of the IL-2R α -chain but remained unchanged after blockade of the β -chain of the IL-2R in the local draining lymph nodes. In addition, we found that both the blockade of the α - and β -chains of the IL-2R down-regulated CD4⁺Foxp3⁺ T cells in the draining lymph nodes. CD4⁺ T cells with reduced proliferation rate in the draining lymph nodes after blockade of the IL-2R β -chain could be T regulatory type 1 cells, Th3 cells producing IL-10 or a mixture of both cell types. Additional experiments are warranted to investigate this possibility.

Blockade of IL-2R β -chain signaling ameliorated AHR and inflammation in experimental asthma, and induced CD4⁺ T cells in the lung producing increased levels of IL-10 and TGF- β but decreased levels of IL-2. TGF- β requires IL-2 to induce CD4⁺CD25⁺Foxp3⁺ T regs from CD4⁺CD25⁺ precursor cells thus explaining why CD4⁺CD25⁺Foxp3⁺ cells are not regulated by blockade of the IL-2R β -chain (34). However, additional experiments such as long-term rechallenge studies and adoptive transfer studies in immune-compromised mice are required to fully investigate the *in vivo* function of CD4⁺CD25⁺Foxp3⁺ cells.

Another finding reported here consists of the selective induction of lung CD4⁺ T cell death (apoptosis) caused by the blockade of CD122. This could be due to growth factor deprivation (IL-2 and IL-15) resulting in expansion of an anergic T cell population producing increased immunosuppressive cytokines that subsequently reduces the proliferation rate without inducing T regs.

Blockade of IL-2R α -chain signaling in experimental asthma led to the suppression of the Th2 cytokines IL-4 and IL-5 as well as down-regulation of T regs as shown by the increased proliferative rate of CD4⁺ T cells isolated from the draining lymph nodes of mice treated with anti IL-2R α -chain Abs. Anti-IL-2R α -chain Abs also had an anti-inflammatory effect in the lung of treated mice and higher doses of these Abs also led to a protective effect on the

AHR to methacholine. The suppression of T cell responses in the regional lymph nodes was critically dependent on the continuous presence of T regs, as local blockade of CD4⁺CD25⁺ T regs by application of Abs against the α -chain of the IL-2R (PC61) showed reduction of their immunosuppressive function. The reversibility of T reg suppression was documented by the induced CD4⁺ T cell proliferation after the blockade of the α -chain of the IL-2R in this model of experimental asthma *in vivo*. These results confirm a previous report showing abrogation of T regs upon application of PC61 Ab in a murine model of asthma (35). However, the present study differs in the application strategy of this Ab, as the Ab was applied here shortly before the challenge phase thereby targeting adaptive T regs. Moreover, we showed here that anti-IL-2R α -chain treatment also targets T effector cells as well as IL-4 and IL-5 production similarly to anti-IL-13 treatment. However, IL-13 production by lung CD4⁺ T cells isolated from these mice was virtually unaffected demonstrating a selective effect of targeting the IL-2R α -chain on Th cytokine production in experimental asthma.

As different T cell subpopulations have been shown to contribute to the AHR in asthma (15, 27–28), we analyzed the cell type that was down-regulated by anti-CD122 Abs. We found that the major cell subpopulation in the lung expressing the β -chain of the IL-2R is the NK cell population that was down-regulated in our experimental model of asthma (36). Whether this subpopulation encloses invariant NKT cells is not known (37). Interestingly, recent data from GATA-3-deficient mice, the main transcription factor for IL-4, IL-5, and IL-13, suggest that iNKT cell development depends on GATA-3 (38). Additional studies are required to address this point. However, we demonstrated in this study that CD8⁺CD122⁺ and CD4⁺CD122⁺ T cells are expanded in experimental asthma. Whereas the capacity of activated CD4⁺ T cells to induce AHR in experimental asthma has been previously described, we recently found that depletion of CD8⁺CD122⁺ T cells also significantly ameliorated AHR in a murine model of asthma (39). Collectively, these data indicate an important role for IL-2R β -chain signaling in controlling T cell function and AHR in experimental asthma. Consistently, treatment of mice with rIL-2 regulated AHR in experimental asthma in the present study. The potential clinical relevance of this observation is highlighted by a recent study by Loppow et al. (40) showing that inhalation of rIL-2 in cancer patients may induce asthma-like symptoms.

In contrast to the blockade of the α -chain, local administration of anti-IL-2R β Abs led to reduced IL-13 but augmented IL-10 and TGF- β production by CD4⁺ lung T cells as well as amelioration of AHR and inflammation at lower doses, suggesting the existence of important IL-2R β -chain-dependent but IL-2R α -chain-independent signaling events in experimental asthma. We thus performed an extensive analysis of the T cells producing IL-13 in experimental asthma and found that the major source of IL-13 production was the CD4⁺ T cell subset and not the NK cells in the lung in this model of asthma. Moreover, we did not find a direct association between cells expressing CD122 and IL-13 production. Furthermore, we obtained no evidence that the IL-13 production in this setting was associated with AHR. The decrease of CD4⁺ T cells producing IL-13 could be an indirect effect of the anti-CD122 Ab treatment because IL-13-producing CD4⁺ T cells do not express CD122 on their surface. As NK T cells are potent producers of IL-13 in asthma (41), subsequent experiments on this T cell subset appear warranted. In any case, the primary effect of Ab treatment is probably due to IL-2/IL-15 deprivation resulting in an anergic T cell population which releases increased IL-10 and TGF- β . These suppressor cytokines may then inhibit production of effector cytokines such as IL-13.

Interestingly, it has been recently demonstrated that the regional lymph nodes are the site of suppression of autoimmune diseases by polyclonal T regs (27). We found here in the OVA model of experimental asthma that local blockade of IL-2/IL-15 β -chain signaling rescues T regs in the draining lymph nodes associated with increased regulatory cytokine production by lung T cells thus suppressing Th2 clones locally. In particular, there was a significant reduction of IL-13 production by lung T cells upon blockade of IL-2R β -chain signaling and inhibition of AHR in experimental asthma. In conclusion, this study provides strong evidence that the IL-2R α -chain and IL-2/IL-15R β -chains are major players in modulating T cell function in experimental asthma. Specifically, IL-2/IL-15R chains control the expansion of T regs in the draining lymph nodes and subsequently the development of pathogenic Th2 cells in the lung in vivo. These findings provide novel insights into the immunopathogenesis of experimental asthma and suggest that targeting these receptor chains may be used for successful therapy of patients with allergic asthma in humans.

Acknowledgments

We thank Prof. J. M. Drazen for critical reading and editing of this manuscript. In addition, we thank M. Schipp and I. Ernst for superb technical help.

Disclosures

The authors have no financial conflict of interest.

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