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Local blockade of IL-6R signaling induces lung CD4+ T cell apoptosis in a murine model of asthma via regulatory T cells

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Abstract

We previously reported high levels of the soluble form of the IL-6R (sIL-6R) in the airways of asthmatic subjects. Here, we analyzed the IL-6R effects on T_h2 cell survival in the lung by locally antagonizing sIL-6R-mediated *trans*-signaling with a designer fusion protein (gp130-Fc) as well as IL-6R signaling with an antibody against the gp80 unit of the IL-6R (αIL-6R) in a murine model of asthma after ovalbumin peptide (OVA) sensitization and challenge. Blockade of the sIL-6R led to a significant decrease in inflammatory cells by an apoptosis-independent mechanism. In contrast, local treatment with αIL-6R antibodies that also block signaling via the membrane-bound IL-6R (mIL-6R) led to decreased signal transducers and activators of transcription (STAT)-3 but not STAT-1 phosphorylation in the lung of treated mice as compared with control-treated mice. Moreover, this treatment induced apoptosis of the cells present in the airways of OVA-treated mice as well as apoptosis of lung CD4+ effector T cells. Subsequent studies showed that this effect was mediated by lung CD4+CD25+Foxp3+ T regulatory cells by a cell–cell interaction, thereby contributing to the resolution of airway hyperresponsiveness in OVA-treated mice given anti-IL-6R antibodies. Taken together, these data suggest that blockade of mIL-6R signaling leads to cell death of lung effector T cells by activating regulatory T cells in experimental asthma. Local targeting of IL-6R signaling could be a novel approach for inducing T_h2 T cell death in allergic airways via regulatory T cells.

Introduction

IL-6 exerts as a pleiotropic cytokine (1) important biological effects on inflammation, immunity and stress (2, 3). Accumulating evidence reveals that IL-6 levels increase in blood (4), bronchoalveolar lavage fluid (BALF) (5) and lung tissues (6) of asthmatic patients. Thus, IL-6 may play an important role in the immunopathogenesis of asthma.

IL-6 binds to the surface IL-6R [membrane-bound IL-6R (mIL-6R)], leading to the dimerization of gp130/IL-6Rβ into tetra- or hexametric structures, thereby forming the active IL-6R complex (7). However, neither IL-6 nor IL-6R alone can bind to or activate gp130. While gp130 is expressed ubiquitously, the expression of mIL-6R is restricted mainly to the membrane of hepatocytes and hematopoietic cells. However, cells lacking the mIL-6R can also respond to IL-6 via the soluble form of the IL-6R (sIL-6R), since the IL-6–sIL-6R complex (hyperIL-6) can activate target cells expressing gp130 in a process termed IL-6 *trans*-signaling (7–10).

Dimerization of gp130 by IL-6 causes the activation of several signaling pathways including the Janus kinase (JAK) and signal transducers and activators of transcription (STAT) pathways. Activation of Jak1, 2 and Tyk2 by IL-6 results in the phosphorylation and activation of STAT-3 and, to much lesser extent, STAT-1 leading to the induction of IL-6 responsive gene expression (11–14). Tyrosine phosphorylation

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leads to homo- or heterodimerization of STAT dimers with subsequent translocation to nucleus where they bind specific DNA sequences in the promoters of target genes. Hereby, IL-6-induced STAT-3 activation is thought to protect cells against apoptosis (15).

We previously demonstrated that allergic patients have an increased baseline expression of the sIL-6R as compared with control subjects. Allergen challenge led to an up-regulation of the sIL-6R associated with an increase of T_h2 CD4+ T cells in the BALF (16). Interestingly, it has been demonstrated that IL-6 derived from antigen-presenting cells (APCs) is able to polarize CD4+ T cells into T_h2 cells (17). In addition, it has been previously shown that IL-6 inhibits the development of T regulatory cells, a subset of T cells important for the control of autoimmunity and allergic diseases. IL-6 thus probably contributes to the development of the allergic diseases by both increasing the number of the effector cells via the soluble component of its receptor and by limiting the protective arm of the immune response via its membrane-bound receptor.

It has been recently demonstrated that IL-23 can differentiate naive CD4+ T cells into pathogenic T_h [T_h17/T_h (IL-17)] that produce IL-17A, IL-17F, IL-6 and tumor necrosis factor-α (18, 19). These cells are known to counteract the naturally occurring CD4+CD25+ T regulatory cells. Consistently, we previously reported that blockade of the mIL-6R in a murine model of asthma led to an increase of the number of adaptive T regulatory cells and decreased the total number of CD4+ T cells in the lung of treated mice. In contrast, blockade of the sIL-6R by application of a chimeric protein gp130-Fc into the airways during the challenge phase led to a down-regulation of the effector CD4+ T cells in this disease without altering the number of T regulatory cells (16). However, the mechanism by which the number of CD4+ T cells decreased after blockade of different components of the IL-6R remained to be elucidated. Here, we investigated the effect of the blockade of IL-6 signal transduction on the survival of inflammatory cells populating the airways in a murine model of asthma. We found that blockade of the IL-6R signal transduction led to increased inflammatory cell death in the airways of anti-IL-6R antibodies-treated mice. In addition, application of the anti-IL-6R antibody in the airways suppressed STAT-3 activation and augmented lung CD4+CD25− T effector cell apoptosis in the presence of CD4+CD25+Foxp3 T regulatory cells in experimental asthma.

Methods

Allergen sensitization/challenge protocol

Female Balb/c mice (6–8 weeks of age) were maintained under specific pathogen-free conditions and received an intraperitoneal (i.p.) injection of 100 μg ovalbumin peptide (OVA) (Calbiochem) complexed with alum (Sigma–Aldrich) on days 0 and 14. On days 25–27, mice underwent anesthesia with avertine (1 mg tribromethanol ml^{-1} t-amylalcohol, 2.5% in PBS) before receiving 50 μg OVA in saline. Control animals received i.p. saline complexed with alum on days 0 and 14 and an intra-nasal (i.n.) dose of 50 μl saline on days 25–27 (16, 20). All experiments were undertaken with approved license (number 177-07-991-32) from the Ethical Committee of the Region Rheinland-Pfalz (Mainz, Germany). All mice were housed in ventilated cages equipped with microisolator lids.

Antibody treatment

To assess the role of IL-6 on airway inflammation, blocking antibodies and control antibodies were given by i.n. administration. Mice received 50 μg of anti-IL-6R antibodies (rat anti-mouse, Chugai Pharmaceutical Inc.), control IgG (rat anti-mouse, Sigma–Aldrich; rat IgG1 isotype control, R&D Systems) or gp130-Fc protein (obtained from K. J. Kallen, University of Kiel, or R&D Systems) by i.n. application on days 24–27. The antibody was given 30 min before the i.n. OVA challenge. On day 28, the bronchoalveolar lavage (BAL) was performed and lungs were used for CD4+ T, CD4+CD25− and CD4+CD25+ cell isolation or frozen at −80°C.

Collection and analysis of the BAL

Twenty-four hours after the last i.n. challenge with either OVA or saline, a BALF of the right lung was performed with 0.75 ml saline for four times (20). Total BALF was collected and cells were stained with trypan blue and counted using a 50 μl aliquot.

Analysis of cell apoptosis in the airways

In order to detect apoptotic cell death in cell cultures were treated with the terminal deoxynucleotidyltransferase that induces incorporation of FITC-labeled deoxynucleotides to DNA strand breaks *in situ*. This reaction is also known as TUNEL technique (*In Situ* Cell Death Detection kit, Fluorescein, Roche Molecular Biochemicals, Mannheim, Germany). Briefly, culture-plate-wells containing cells were fixed with for 60 min with 4% PFA and incubated with permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 2 min at 4°C. After incubation with TUNEL reaction mixture for 1 h at 37°C, the reaction was stopped by incubation with water. Negative controls were performed by applying 50 μl of the label solution instead of the TUNEL reaction mixture to the cytospins. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole staining (Vector Mounting Medium, Vector Laboratories, Burlingame, CA, USA) and analyzed by a fluorescence microscope (Olympus).

Isolation and analysis of lung and spleen CD4+ and lung CD4+CD25+ and CD4+CD25− T cells

Lungs were removed from mice, transported in DMEM and minced into small pieces (1–2 mm^2) using a forceps. Tissue pieces were suspended in Dulbecco's PBS (1 lung equivalent = 5 ml) containing 300 U ml^{-1} of collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 0.001% DNase (Roche Diagnostics, Heidelberg, Germany) to obtain a cell suspension as previously described (16, 21). The lung cell suspensions were then incubated with anti-CD4 beads (10^7 Dynabeads Mouse CD4-L3T4 ml^{-1}; 25 μl beads to isolate 2.5×10^6 cells) for 20 min at 4°C under shaking conditions. CD4+ T cells were then isolated by positive selection by using a magnet in accordance to the

manufacturer's instructions (Dynal, Hamburg, Germany). Afterward, the anti-CD4 beads were detached by using mouse CD4 DETACHaBEAD (Dynal). Briefly, 10 μl of mouse CD4 DETACHaBEAD were incubated with 1×10^6 CD4+ T cells under rotating conditions for 45 min at room temperature. In additional experiments, the resulting CD4+ lung cells (purity 97%) were further separated by cell sorting into CD4+CD25+ and CD4+CD25− T cells. Briefly, isolated lung CD4+ T cells from Balb/c OVA-sensitized and -challenged (untreated), anti-IL-6R antibody-treated and IgG-treated mice were stained with 1 μg CD25–PE/1×10^6 (clone PC61, BD Biosciences) cells at 4°C for 30 min in the dark. Cells were then washed once in PBS and kept on ice. CD4+CD25+ cells were sorted on a BD FACSVantage SE System, and analysis was done with CellQuest Pro software (BD Biosciences). Dead cells and debris were excluded from data analysis through appropriate forward scatter channel and side scotter channel (SSC) gating. For each sample, 20 000 events were collected. Markers and regions were set according to control staining. In some experiments performed in duplicate, sorted lung CD4+CD25+ cells (10^5 cells per well) or CD4+CD25− lung cells (10^5 cells per well) were cultured overnight with 2 μg ml^{-1} of soluble anti-CD3 antibodies (BD Biosciences; PharMingen, San Diego, CA, USA).

For apoptosis studies with Annexin V–propidium iodide (PI), after three washes in complete medium, the isolated lung CD4+ T cells were set-up in culture with plate-bound anti-CD3 alone or together with soluble anti-CD28 antibodies (CD3, clone 145-2C11, 5 μg ml^{-1}; CD28, clone 37.51, 2 μg ml^{-1}; PharMingen) for 24 h at a concentration of 1×10^6 cells ml^{-1}. Cell pellets were processed for cell apoptosis using the Annexin V–PI staining (Annexin V–FITC apoptosis detection kit II, PharMingen, Becton Dickinson). Briefly, CD4+ cells were washed in cold PBS and re-suspended in binding buffer. Cells (10^5) were then incubated either with Annexin V–FITC or –PI or with both and analyzed within 1 h by FACS analysis. In some samples, TUNEL assay was performed as specified above.

Protein extraction and western blot analysis

Tissue proteins were extracted as previously described (21). Briefly, tissue was homogenized in PBS and protein extracted in the presence of protease inhibitors (6.75% aprotinin and 312 μg ml^{-1} trypsin inhibitors) and detergent (Nonidet). Protein concentrations were determined by spectrophotometry with a Bio-Rad protein assay (Bio-Rad Laboratories, München, Germany). The standard curve was performed by using 0, 4, 8 and 16 μg of BSA ml^{-1}. Equal amounts of protein extract were added to electrophoresis sample buffer. After boiling total lung proteins were separated by 10 or 15% SDS–PAGE, transferred to nitrocellulose membranes and incubated in blocking solution (5% milk in PBS/0.05% Tween 20) for 1 h at room temperature, and subsequently exposed to 1 μg ml^{-1} of antibody against either p-Tyr (mouse mAb for the immunoprecipitated STAT-3 and STAT-1) or β-actin (goat polyclonal antibody) (Santa Cruz, Heidelberg, Germany) in blocking solution. Specific binding was visualized with the enhanced chemiluminescence (ECL) western blotting detection system according to the manufacturer's instruction after 1 h incubation with the corresponding secondary antibody horseradish peroxidare (HPR) conjugated; 1:2000 in blocking solution) (Amersham Pharmacia Biotech, Germany).

Immunoprecipitation

For immunoprecipitation, 250 μg total lung proteins were pre-cleared with 1 μg of appropriate IgG according to the primary antibody and 20 μl A/G plus agarose (Santa Cruz) for 30 min at 4°C. After centrifugation at 2500 r.p.m. for 5 min, the supernatant was collected and incubated with 2 μg primary antibody (STAT-3 or STAT-1) for 1 h at 4°C followed by addition of 20 μl 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 four times with PBS and finally re-suspended in 50 μl PBS. A western blot was performed as described before (16) with 12 μl of precipitated protein for STAT-1/P-STAT-1 and STAT-3/P-STAT-3 detection as described above. Briefly, after being boiled, proteins were separated by 10 or 15% SDS–PAGE, transferred to nitrocellulose membranes and detected with a specific antibody against p-Tyr residues (Santa Cruz). Specific binding was visualized with the ECL western blotting analysis system according to the manufacturer's instructions after 1 h incubation with the corresponding secondary HPR-conjugated antibody (1:2000 in blocking solution, Amersham Biosciences).

Isolation of RNA from lung CD4+ T cells

Sorted and cultured lung CD4+ T cells were immediately frozen after culture until RNA was isolated according to the manufacturer's instructions (RNeasy Micro Kit, QIAGEN). Briefly, cells were disrupted directly in the plastic wells by adding 350 μl of lysis buffer containing β-mercaptoethanol. Cells were then homogenized with a rotor for 30 s and mixed with an equal volume (350 μl) of 70% ethanol. The samples were then applied to an RNeasy MinElute Spin Column (QIAGEN) in a 2 ml collection tube. Closed tubes were then centrifuged for 15 s at $8\,000 \times g$. The column was washed several times, and the eluate was collected by applying 14 μl of RNase-free water to the column. RNA concentration was determined by densitometry.

Reverse transcription followed by real-time PCR analysis of Foxp-3 mRNA of CD4+ T lung cells

Six microliters of total RNA from lung CD4+ T cells were then reverse transcribed by the use of the RevertAid first strand cDNA synthesis kit for reverse transcription (RT)–PCR (M-murine leukemia virus RT, MBI Fermentas GmbH). The resulting cDNA was then used as a template for the real-time PCR.

To eliminate amplifications from contaminating genomic DNA, the following primers were designed to span an intron/exon boundary and thus to anneal specifically to cDNA: FoxP3, forward 5′-CTTATCCGATGGGCCATCCTGGAAG-3′ and FoxP3, reverse 5′-TTCCAGGTGGCGGGGTGGTTTCTG-3′ and HGPRT, forward 5′-GTTGGATACAGGCCAGACTTTGTTG-3′ and HGPRT, reverse 5′-GAGGGTAGGCTGGCCTATAGGCT-3′.

Real-time analysis of FoxP3 mRNA was performed on an iCycler (Bio-Rad Laboratories) using the IQ SYBR Green Supermix (Bio-Rad Laboratories). After normalization of the data according to the expression of Hypoxanthin-Guanin phosphoribosyl transferase (HGPRT) mRNA, relative expression levels of Foxp3 mRNA were calculated.

Statistical analysis

Differences were evaluated for significance by the Student's two-tailed *t*-test for independent events (Excel, PC). The coefficient of correlations was calculated by using the statistical analysis of the Excel program. Data are given as mean values ± SEMs.

Results

I.n. administration of anti-IL-6R antibodies suppresses STAT-3 activation in the airways in a murine model of asthma

We have previously demonstrated that blockade of IL-6 signal transduction in the airways by i.n. application of anti-IL-6R antibodies during the challenge phase induced amelioration of airway hyperresponsiveness (AHR) in a murine model of asthma (16). Since IL-6 is known for its anti-apoptotic effect on hematopoietic cells such as B and T cells, we asked whether anti-IL-6R antibodies given i.n. would potentially lead to the resolution of AHR by inducing apoptosis of inflammatory cells in the airways of treated mice by means of T regulatory cells. In these studies, we used the experimental protocol depicted in Fig. 1. To demonstrate the effects of anti-IL-6R antibody treatment on the IL-6-inducible transcription factor STAT-3 in the airways, we determined STAT-3 phosphorylation by immunoprecipitation of total lung proteins. As shown in Fig. 2(A), after immunoprecipitation of total lung proteins with an antibody against STAT-3 followed by western blot analysis for phospho-tyrosine residues, anti-IL-6R antibody treatment delivered i.n. during the challenge phase significantly decreased STAT-3 phosphorylation in the lungs in a murine model of allergic asthma after OVA sensitization as compared with OVA-sensitized, untreated and IgG control-treated mice in two independent experiments (Fig. 2A and B). In contrast, specific blockade of the sIL-6R via i.n. application of the fusion protein gp130-Fc did not significantly suppress the levels of P-STAT-3 in this model of asthma (Fig. 2C and D) suggesting that signaling via the mIL-6R rather than the sIL-6R controls STAT-3 activation in experimental asthma.

I.n. administration of anti-IL-6R antibodies but not gp130-Fc protein increases cell death in the BALF

As STAT-3 activation has been reported to prevent cell death, we analyzed in a subsequent series of experiments the effect of local blockade of IL-6 signaling on cell death in the BALF of OVA-sensitized and -challenged mice. Anti-IL-6R antibody treatment led to an increased number of dead cells in the airways, as demonstrated by trypan blue exclusion staining of cells obtained from the airways of treated mice (Fig. 3, upper panel). Consistent with the increased cell death in the airways, the number of viable inflammatory cells

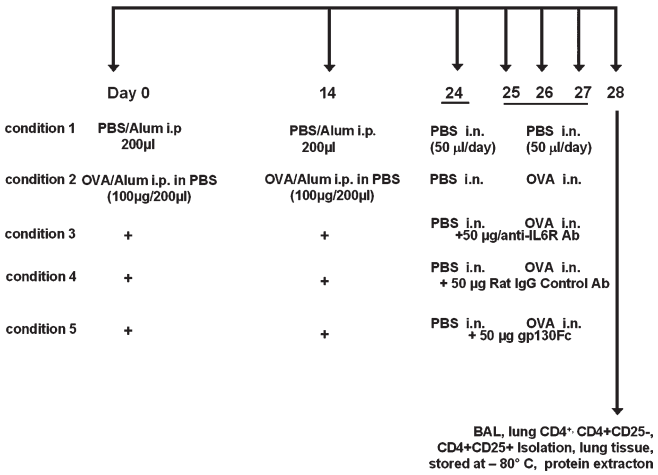

Fig. 1. Experimental design of local anti-IL-6R antibody and gp-130-Fc treatment in a murine model of asthma. Female Balb/c mice (6–8 weeks of age) were maintained under specific pathogen-free conditions and received i.p. injections of 100 µg OVA (Calbiochem) complexed with alum on days 0 and 14 (conditions 2–5). On days 25–27, mice underwent anesthesia with avertine before receiving 50 µg OVA in saline. In addition, some mice received 50 µg of anti-IL-6R antibodies (condition 3), the fusion protein gp130-Fc (condition 5) or rat IgG (condition 4). Control animals received i.p. saline complexed with alum on days 0 and 14 and an i.n. dose of 50 µl saline on days 25–27 (condition 1). To assess the role of IL-6 on airway cell survival, blocking antibodies and control antibodies were given by i.n. administration. Mice received 50 µg of anti-IL-6R antibodies (rat anti-mouse, Chugai Pharmaceutical Inc.) and control IgG (rat anti-mouse, Sigma–Aldrich; rat IgG1 isotype control, R&D Systems) by i.n. administration on days 25–27. The antibody was given 30 min before the i.n. OVA challenge. On day 28, BAL was obtained and lung tissues were either stored for further analysis or immediately processed for CD4+ T cell isolation, as described in the Methods.

in the airways of anti-IL-6R-treated mice was significantly reduced indicating an ongoing anti-inflammatory effect in the airways by inhibition of IL-6R signaling (Fig. 3, lower panel). In contrast, selective blockade of the sIL-6R via i.n. application of the designer fusion protein gp130-Fc did not show induction of cell death in the airways. However, the number of viable cells was significantly decreased suggesting that sIL-6R signaling modulates cell migration rather than cell viability into the lung (Fig. 3, lower panel).

Anti-IL-6R antibody treatment induces CD4+ T cell apoptosis in a murine model of asthma

It has been previously reported that IL-6 signaling leads to increased spleen CD4+ T cell survival. Using a time course experiment, we assessed the effects of recombinant IL-6 on T cell survival and observed that IL-6 induces spleen CD4+ T cell survival at 72 h after beginning of the cell culture. IL-6 in fact was able to suppress the number and percentage of apoptotic T cells in culture (Fig. 4A–C). Co-incubation with anti-IL-6R antibodies but not an irrelevant IgG antibody neutralized the late anti-apoptotic effect of IL-6 on spleen CD4+ T cells, as shown by Annexin V and PI staining (Fig. 4A–C). While Annexin V staining alone represents early stages of apoptosis involving perturbation of the phospholipids in the cellular membrane, double-positive Annexin V–PI staining as shown in Fig. 4(A–C) represents late apoptotic cells.

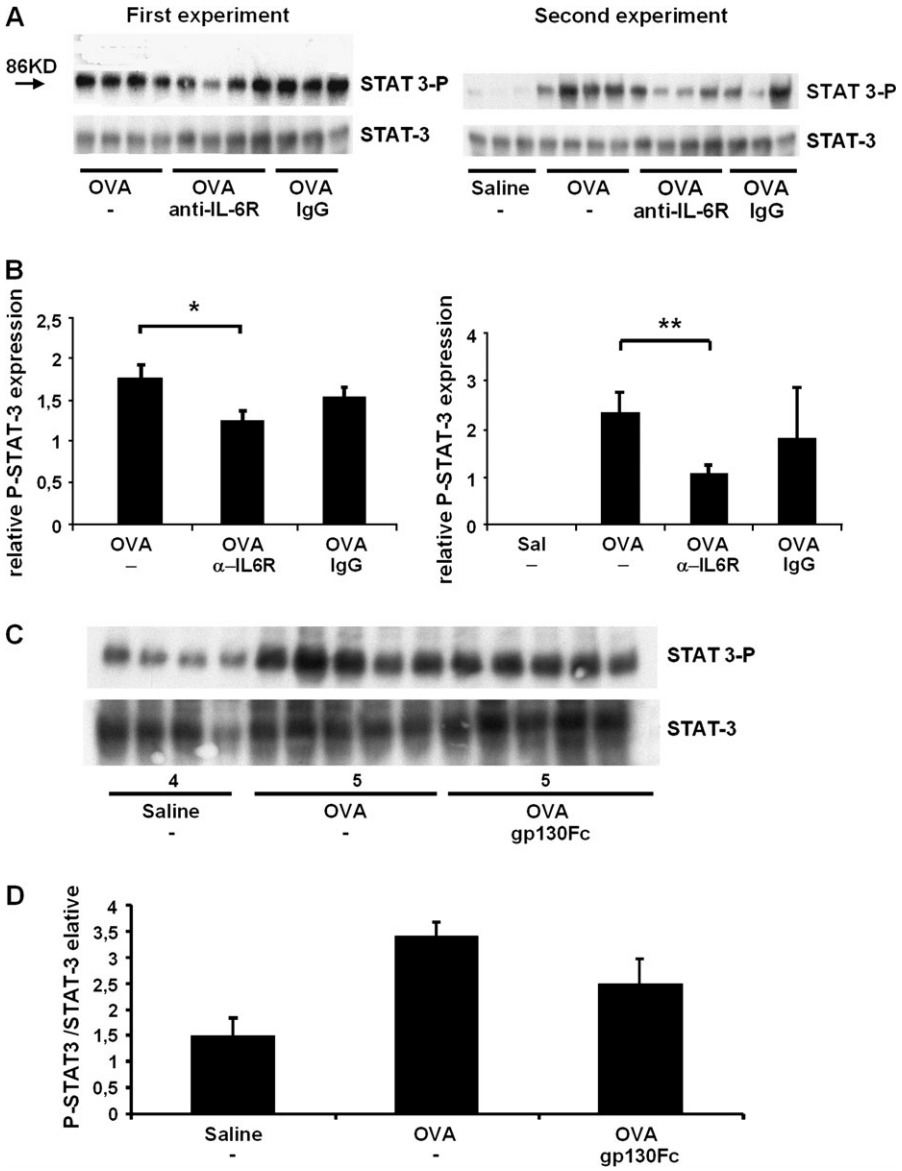

Fig. 2. Blockade of STAT-3 activation in the airways of mice treated with anti-IL-6R antibodies. i.n. delivery of antibodies to the IL-6R causes a decrease of STAT-3 phosphorylation in the lung, as shown by immunoprecipitation of total lung protein with an antibody against STAT-3 followed by western blot analysis for phospho-tyrosine 705 residues in two independent experiments (A). As total STAT-3 levels were unaffected by anti-IL-6R antibody treatment, this led to a significant decrease in the P-STAT-3/STAT-3 ratio (B). In few single mice, total IgG led to reduced pSTAT3 levels, probably due to the well-known anti-inflammatory properties of a minor species of IgGs that is modified with terminal sialic acids on their Fc-linked glycans (29). In contrast to the effects of anti-IL-6R antibodies, however, this IgG effect on average did not reach statistical significance (A and B). In contrast to anti-IL-6R antibody therapy, P-STAT-3 levels were not changed upon gp130-Fc treatment (C and D). Quantitative analysis was performed by using a Biometra software system for STAT-3 (B and D). The number of lung from different mice is shown for each blot as *n*. Mean values ± SEMs are indicated. Statistically significant differences between the OVA anti-IL-6R and OVA IgG groups are highlighted by an asterisk (*$P < 0.05$ and **$P < 0.01$).

Based on these data showing that IL-6 signaling controls T cell apoptosis in peripheral T cells, we next tested the possibility that local treatment with anti-IL-6R antibodies in experimental asthma leads to increased CD4+ T cell apoptosis in the lung. Accordingly, we isolated CD4+ T cells from the lung of different experimental groups and analyzed CD4+ T cell apoptosis after overnight stimulation with anti-CD3/anti-CD28 antibodies. As shown in Fig. 5(A), after Annexin V and PI staining, the number of apoptotic CD4+ T cells was significantly increased after *in vivo* treatment with anti-IL-6R antibodies as compared with the control OVA IgG treatment. In contrast, local blockade of the sIL-6R by i.n. application of the fusion protein gp-130-Fc did not lead to significant induction of programmed cell death of lung CD4+ T cells (data not shown).

Taken together, we concluded that blockade of mIL-6R rather than sIL-6R signaling in lung CD4+ T cells leads to increased apoptosis of T cells and other inflammatory cells in

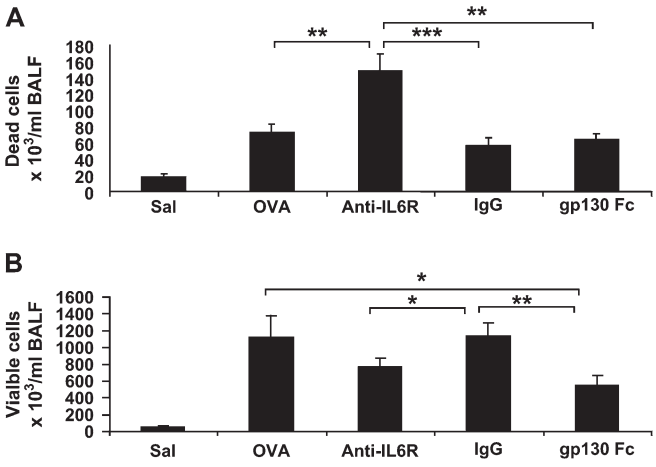

Fig. 3. Increased programmed cell death of inflammatory cells in the airways of anti-IL-6R antibody-treated mice. Experimental asthma is characterized by an increased number of inflammatory cells in the airways, most of them (70%) being eosinophils. In all four experimental groups, staining with the exclusion dye trypan blue was performed to identify dead cells in the BALF. As shown, the number of dead cells decreased after OVA sensitization as compared with saline-treated mice. Moreover, OVA-sensitized and -challenged mice that received i.n. anti-IL-6R antibody treatment had a significantly ($P < 0.001$) increased number of dead cells in the BALF as compared with those receiving IgG antibodies or the fusion protein gp130-Fc. Moreover, the total cell number of viable cells retrieved from the lavage of anti-IL-6R antibody-treated mice was significantly reduced compared with IgG-treated mice indicating suppression of airway inflammation by this treatment. Eight mice per group were analyzed in this representative experiment. At least three more experiments were performed.

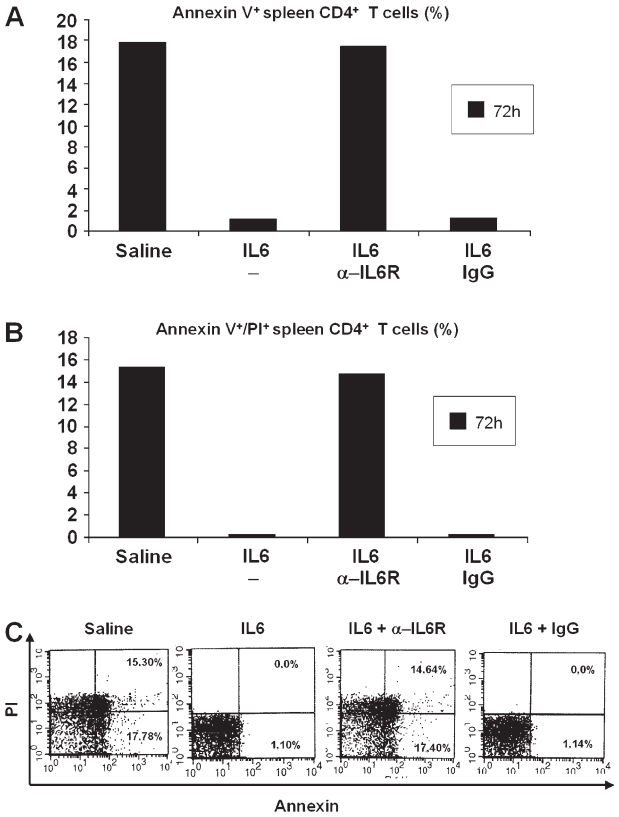

Fig. 4. IL-6 rescues spleen CD4+ T cells from apoptosis and anti-IL-6R antibody treatment neutralizes this effect in cell culture. Naive CD4+ spleen T cells were purified and placed in culture for 72 h with anti-CD3 and anti-CD28 antibodies. To test the effect of IL-6 on CD4+ T cell survival, we added recombinant IL-6 (10 ng ml^{-1}) alone or in conjunction with anti-IL-6R antibodies or IgG control antibody (10 μg ml^{-1}). After 72 h of cell culture, CD4+ T cells were harvested and stained with Annexin V and PI for subsequent FACS analysis. The percentage of early apoptotic cells with perturbations at the lipid layer (Annexin V+/PI−) is shown in panel (A), while the percentage of late Annexin V plus PI-positive cells is shown in panel (B). IL-6 caused a marked reduction in the percentage of early and late apoptotic cells that could be reversed by the addition of anti-IL-6R antibodies. In panel (C), representative dot blots from one spleen are shown.

the airways thereby probably contributing to the previously reported improvement of the AHR upon anti-IL-6R antibody therapy. Moreover, gp-130-Fc application did not cause T cell apoptosis.

Increased T regulatory cells present in the lung CD4+ T cell pool contribute to the CD4+ T effector cell death after in vivo application of anti-IL-6R antibodies

We thus asked the question whether the increased T regulatory cells that we previously described upon i.n. application of anti-IL-6R antibody but not gp130-Fc (16) would be responsible of the increased apoptosis rate seen in lung CD4+ T cells. Accordingly, we first performed real-time PCR for Foxp-3 in isolated lung CD4+ T cells and found that anti-IL-6R antibody treatment induces Foxp-3 as compared with gp130-Fc or OVA-untreated mice, as determined by real-time PCR (Fig. 5B) demonstrating that the T regulatory cells did not undergo apoptosis after anti-IL-6R treatment. This point was confirmed on isolated lung CD4+CD25+ and CD4+CD25− T cells. In fact, we could demonstrate that administration of anti-IL-6R antibodies had no effects on apoptosis of lung CD4+CD25+ regulatory T cells (Fig. 5C). Interestingly, such administration also had no direct effect on the apoptosis of lung CD4+CD25− effector cells when cultured alone, suggesting an indirect, potentially contact-dependent mechanism through which mIL-6R signaling controls apoptosis of lung effector T cells (Fig. 5C). Accordingly, we set-up co-incubation experiments with lung effector T cells and regulatory T cells. Indeed, co-incubation of lung CD4+CD25− effector cells with CD4+CD25+ T regulatory cells induced apoptosis in the CD4+CD25− effector population (Fig. 5C and D) indicating a causative effect between addition of T regulatory cells and the induction of apoptosis of the effector cells. Therefore, our data suggest that mIL-6R signaling controls apoptosis of effector CD4+CD25− T cells in experimental asthma by modulating the effector function of regulatory T cells.

Discussion

Effector T_h2 cells play an important role in the development of atopic diseases. In atopic asthma, peripheral T cells are primed to produce a 2-fold higher basal level of T_h2 cytokines compared with healthy controls, which is further enhanced after allergen challenge (22). Although it is known that cytokines are crucial for the differentiation toward a T_h1

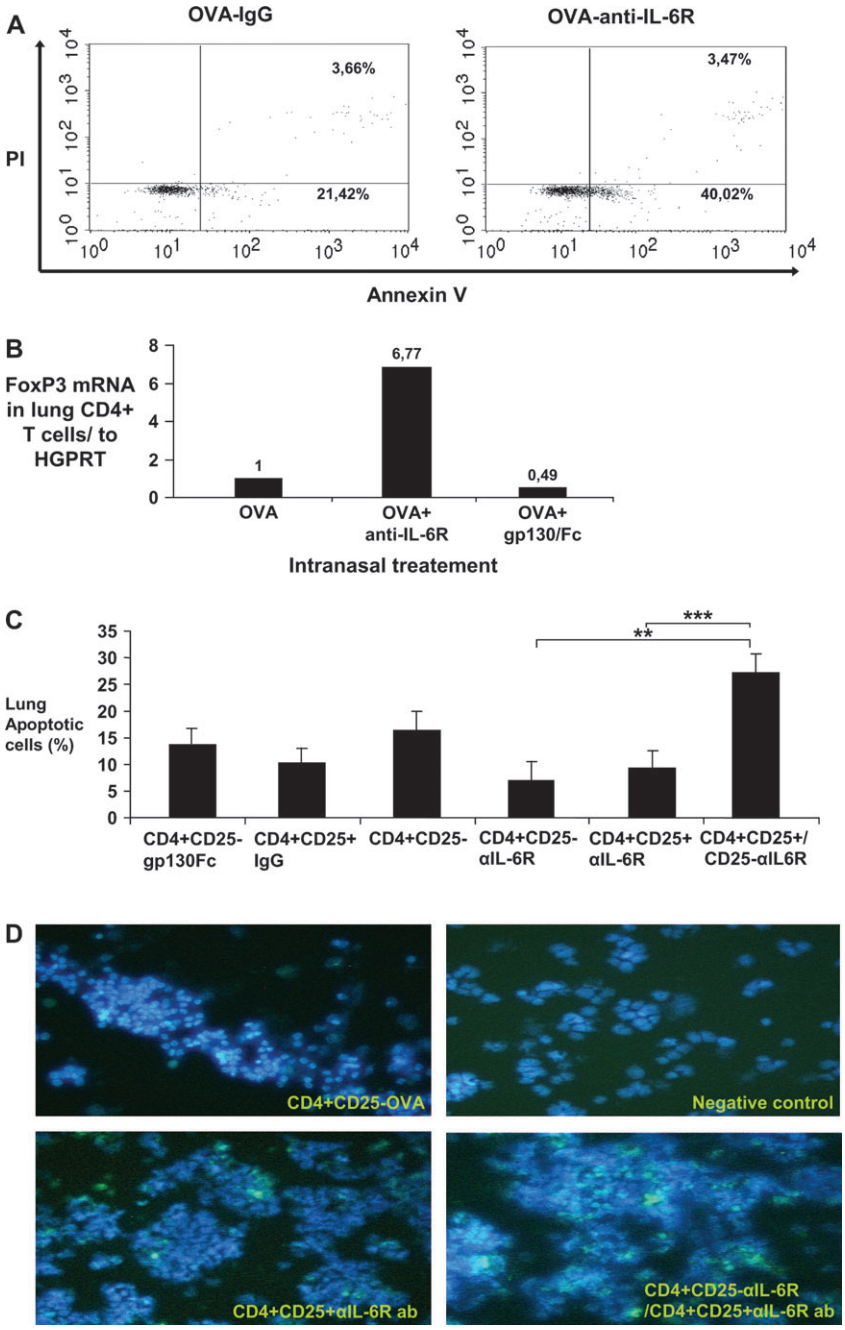

Fig. 5. Application of anti-IL-6R antibodies in the airways induces lung CD4+CD25− T effector cell apoptosis via CD4+CD25+ Foxp3+ T regulatory cells. (A) To investigate the effect of the anti-IL-6 antibody treatment on pulmonary CD4+ T cells, lung T cells were isolated and subjected to FACS analysis using Annexin V–PI staining. It was found that anti-IL-6R antibody treatment induced lung CD4+ T cell early apoptosis as compared with IgG treatment during the challenge phase in a murine model of asthma. One representative dot blot from 7 to 12 mice per group is shown. (B) To answer the question whether T regulatory cells or T effector cells would undergo apoptosis after anti-IL-6R antibodies treatment, we analyzed Foxp-3 expression in isolated lung CD4+ T cells and found that T regulatory cells were increased in the pool of total CD4+ T cells isolated from the lung of anti-IL-6R antibody-treated mice indicating apoptosis in the pool of lung CD4+ relates to the effector T cells, as shown by real-time PCR. (C) In additional experiments, we analyzed the number of apoptotic CD4+CD25− as well as CD4+CD25+ T cells when cultured alone or in co-culture. As shown in (C), the apoptotic rate of CD4+CD25+ and CD4+CD25− T cells was similar and regardless on the treatment they were exposed to for the previous 3 days *in vivo*. However, when the two populations (CD4+CD25+ and CD4+CD25−) were cultured together after isolation in the presence of anti-IL-6R antibodies, the apoptotic rate as determined by TUNEL assay was significantly induced. (D) Example of TUNEL assay of the different CD4+ cell populations treated *in vivo* as indicated. Pictures were taken at ×200 with a digital camera on an Olympus Microscope and imported on a computer, saved and later on 20–40 pictures were analyzed by counting 4′,6-diamidino-2-phenylindole-positive (blue) and TUNEL-positive nuclei. Two experiments were performed and 200–500 cells per condition were analyzed by two independent investigators. Results in panel (A) are reported as % of TUNEL-positive cells/total cells/picture.

or T_h2 phenotype, the mechanisms by which gene transcription and differentiation are regulated remain largely unclear. Here, we demonstrate a striking effect of the pro-inflammatory cytokine IL-6 (23) in enhancing the *in vivo* expansion and survival of antigen-stimulated CD4+ T cells in a murine model of asthma. In fact, blockade of the IL-6 signal transduction by i.n. application of an antibody blocking both the mIL-6R and the sIL-6R resulted in an increased CD4+ T cell apoptosis in the lung of treated mice. These results give novel insights into IL-6R signaling in experimental asthma.

During infections, recognition of microbial molecules by toll-like receptors (TLRs) results in activation of APCs. The APCs then produce soluble factors that together override the suppressive effects of regulatory T cells and allow efficient expansion of T effector cells against pathogens (24). IL-6 may play a pivotal role in this context, as it protects murine splenic CD4+ T cells stimulated with immobilized anti-CD3 and anti-CD28 antibodies from apoptosis. Similarly, IL-6 has been reported to enhance *in vitro* survival of human CD4+ T cells that had been pre-activated *in vivo* (23). As alveolar macrophages from atopic asthmatics produce higher levels of IL-6 than alveolar macrophages from healthy controls, IL-6 produced at the sites of tissue inflammation in asthma may activate local lymphocytes for enhanced production of T_h2 cytokines and provide an important anti-apoptotic stimulus for their survival.

In this study, we demonstrate a key role of IL-6 signal transduction for cytokine production of T cells in the effector phase of a murine asthma model induced by OVA. Specifically, we found that local application of anti-IL-6R antibodies leads to a decreased STAT-3 phosphorylation in the lung of treated mice. This effect of IL-6 in promoting survival is not unique to T cells. A variety of other cell types have been shown to survive better in the presence of IL-6, including mast cells (25). Although the direct effect of IL-6 as a survival factor has been shown directly in many cell types including CD4+ T cells, we cannot exclude the possibility that some of its effects on the enhanced expansion of activated CD4+ T cells are mediated indirectly by restraining the inhibitory activity of the regulatory CD4+CD25+ T cells *in vivo*, as proposed by Pasare and Medzhitov (24).

Depending on the cell type and the mode of cell activation, STAT transcription factors can mediate either pro- or anti-apoptotic signals. However, STAT-3 and STAT-5 have mainly been implicated in promoting cell survival (26–28). We previously reported that specific blockade of both mIL-6R and sIL-6R in the lung leads to reduction of CD4+ T cells in the lung. Here, we found that this observation is related to the induction of lung CD4+ T cell apoptosis associated with suppression of STAT-3 activation upon blockade of signaling via the mIL-6R rather than the sIL-6R. STAT proteins may mediate transcriptional activation of genes that are involved in induction of apoptosis (27). IL-6-induced STAT-3 activation may either directly augment lung CD4+ T cell survival in asthma or activate expression of anti-apoptotic genes.

Finally, our data demonstrate that signaling via the mIL-6R rather than the sIL-6R controls the effector functions of lung regulatory T cells in experimental asthma. Specifically, we used in these studies the designer fusion protein gp130-Fc that blocks signaling via the sIL-6R but not the mIL-6R (9, 10). The blockade of the sIL-6R via gp130Fc had no effect on STAT-3 activation and apoptosis of lung CD4+ T cells and a previous study showed that such blockade (in contrast to mIL-6R blockade) also had no effect on the induction of T regulatory cells in experimental asthma (16). Therefore, our results suggest that STAT-3 activation in the lung is mainly determined by the function of the mIL-6R on regulatory T cells and that blockade of the sIL-6R is not sufficient (in the presence of mIL-6R signaling) to effectively inhibit STAT-3 activation in experimental asthma. The present findings also indicate that blockade of mIL-6R signaling not only induces an expansion in the number of lung regulatory T cells (16) but also critically modulates their function. In fact, co-culture studies suggested that blockade of IL-6R signaling enables regulatory T cells to induce cell death of lung effector T cells. These data provide novel insights into the mechanism of action of regulatory T cells in asthma and are consistent with the recent demonstration that regulatory T cells may kill effector T cells in other diseases via apoptosis. In any case, these data suggest the potential utility of antibodies against the IL-6R as a novel molecular approach for targeting STAT-3-dependent lung T cell survival and for the treatment of patients with allergic asthma in humans.

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Abbreviations

AHR	airway hyperresponsiveness
APC	antigen-presenting cell
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
ECL	enhanced chemiluminescence
Foxp3	forkhead winged-helix
HPR	horseradish peroxidase
HGPRT	hypoxanthin-guanin phosphoribosyl transferase
i.n.	intra-nasal
i.p.	intra-peritoneal
JAK	Janus kinase
mIL-6R	membrane-bound IL-6R
OVA	ovalbumin peptide
PI	propidium iodide
RT	reverse transcription
sIL-6R	soluble form of the IL-6R
STAT	signal transducers and activators of transcription
TUNEL	terminal deoxynucleotidyltransferase-mediated fluorescein-dUTP nick end labeling

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