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Induction of tolerogenic lung CD4$^+$ T cells by local treatment with a pSTAT-3 and pSTAT-5 inhibitor ameliorated experimental allergic asthma

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Abstract

Signal transducer and activator of transcription (STAT)-3 inhibitors play an important role in regulating immune responses. Galiellalactone (GL) is a fungal secondary metabolite known to interfere with the binding of phosphorylated signal transducer and activator of transcription (pSTAT)-3 as well of pSTAT-6 dimers to their target DNA *in vitro*. Intra nasal delivery of 50 μg GL into the lung of naive Balb/c mice induced FoxP3 expression locally and IL-10 production and IL-12p40 in RNA expression in the airways *in vivo*. In a murine model of allergic asthma, GL significantly suppressed the cardinal features of asthma, such as airway hyperresponsiveness, eosinophilia and mucus production, after sensitization and subsequent challenge with ovalbumin (OVA). These changes resulted in induction of IL-12p70 and IL-10 production by lung CD11c$^+$ dendritic cells (DCs) accompanied by an increase of IL-3 receptor α chain and indoleamine-2,3-dioxygenase expression in these cells. Furthermore, GL inhibited IL-4 production in T-bet-deficient CD4$^+$ T cells and down-regulated the suppressor of cytokine signaling-3 (SOCS-3), also in the absence of STAT-3 in T cells, in the lung in a murine model of asthma. In addition, we found reduced amounts of pSTAT-5 in the lung of GL-treated mice that correlated with decreased release of IL-2 by lung OVA-specific CD4$^+$ T cells after treatment with GL *in vitro* also in the absence of T-bet. Thus, GL treatment *in vivo* and *in vitro* emerges as a novel therapeutic approach for allergic asthma by modulating lung DC phenotype and function resulting in a protective response via CD4$^+$FoxP3$^+$ regulatory T cells locally.

Keywords: experimental asthma, FoxP-3, IDO, SOCS-3, STAT-3, STAT-5, STAT-6, tolerance

Introduction

Dendritic cells (DCs) control the activation and maturation of CD4$^+$ T helper (T_h cells) and determine key steps in innate and adaptive immunity (1, 2). Current therapies for allergic asthma enclose glucocorticoids that suppress inflammatory cytokines, (3) such as IL-6 and IL-4, released by antigen-presenting cells and NK cell subsets (4–8). Steroid-induced immune tolerance has beneficial effects on allergic asthma via Toll-like-receptor-mediated reduced DC maturation (9). Whether DCs are immunostimulatory, anergic or tolerogenic depends on their expression of regulatory surface molecules and intracellular transcription factors defining their immunopathogenic character mediated by the cytokines they release.

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The transcription factor signal transducer and activator of transcription (STAT-3), a protein induced and phosphorylated via IL-6 or IL-10 (10) cytokine signaling, controls activation and function of $CD4^+$ T cells by tolerogenic DCs (11) induction and is critical for the development of regulatory T (T reg) cell populations (12). It has been previously demonstrated that galiella-lactone (GL), a fungal secondary metabolite isolated from the ascomycete *Galiella rufa* (13), inhibited STAT-3-dependent (IL-6) and STAT-6-dependent (IL-4) reporter gene expression with high selectivity resulting in decreased activated STAT-3 dimer binding to the target promoter (14). Since suppressor of cytokine signaling-3 (SOCS-3) proteins (15) are known to be inhibitory to IL-6/STAT-3 signaling and to regulate onset and maintenance of T_h2-mediated allergic responses, we reasoned that the decrease of activated lung STAT-3 binding on its target DNA (16) would interfere with SOCS-3 expression.

Consistently, intra-nasal application of GL decreased lung T_h2 cells, lung IL-17A-producing cells, while it induced T_h1 cells, the expression of indoleamine-2,3-dioxygenase (IDO) and regulatory $CD4^+$ T cells in the lung. GL-mediated T-cell responses were accompanied by inhibition of STAT-3–STAT-5 DNA binding/activation resulting in increased anti-inflammatory cell subsets expansion.

In conclusion, GL treatment ameliorated experimental asthma by inducing a tolerogenic milieu by changing the DC subsets (17) by STAT-3 inhibition, thus providing a new possible therapeutic approach for this disease.

Methods

Mice

Balb/cJ mice were purchased from the animal facility of the Universitätsmedizin Mainz. T-bet-deficient mice were kindly provided to us by Prof. Laurie Glimcher, Harvard Medical School, Boston, MA. T-cell-specific STAT-3-deficient mice were generated by Prof. K. Takeda in the laboratory of Prof. S. Akira who kindly provided them to us. These mice were previously described (18).

Producing organism, fermentation and isolation of GL

GL was purified from the ascomycete IBWFA111-95 isolated from wood (13). The strain was kindly provided by Prof. H. Anke and is deposited in the culture collection of the Institute for Biotechnology and Drug Research (IBWF e.V., Kaiserslautern, Germany). For maintenance on agar slants, the strain was kept on YMG medium composed of yeast extract 0.4%, malt extract 1%, glucose 1%, pH 5.5, and agar 1.5% for solid media. Fermentations were carried out in a Braun Biostat A-20 fermenter containing 20 l of YMG medium with aeration (3 l air min^{-1}) and agitation (120 r.p.m.) at 22°C. The production of GL was followed by analyzing mass and ultraviolet (UV) spectra of daily culture fluid samples (50 ml) extracted with an equal volume of ethyl acetate (EtOAc), evaporated to dryness and resolved in methanol at a concentration of 5 mg ml^{-1}. The mass and UV spectra were analyzed with a Hewlett–Packard Series 1100LC-MSD instrument fitted with a LiChroCART Superspher 100 RP-18 column (125 × 2 mm, 4-mm particle size; Merck, Darmstadt, Germany). The chromatographic conditions consisted of a gradient from 1 to 100% acetonitrile in 20 min and an isocratic step at 100% acetonitrile for 1 min at 40°C and 10 μl injection volume was used. The flow rate was 0.45 ml min^{-1}. The fragmentor voltage was set to 140 V in the positive and negative atmospheric pressure chemical ionisation (APCI) modes. The spectra obtained were compared with the reference library of the IBWF e. V.. After 700 h of fermentation, the culture fluid was separated from the mycelium by filtration and extracted with EtOAc. The solvent was evaporated and the crude product (2.3 g) was separated by chromatography on silica gel (Merck 60) with cyclohexane:EtOAc (70:30) as eluant resulting in 1.2 g of an enriched product. Preparative HPLC (Macherey–Nagel Nucleosil 100-7 C-18, column 40 × 250 mm) with water:MeOH (46:54) as eluant yielded 635 mg of GL. The purity of the isolated GL was analyzed by HPLC-DAD/MS using the conditions described above. GL showed the highly characteristic fragmentation pattern in the APCI-positive mass spectrum revealing the molecular ion (Fig. 1A). The purity of GL as estimated by HPLC-DAD/MS analysis was >97.5% (Fig. 1A).

The naive compound is resistant to heat or other inactivation methods in comparison with proteins and it has passive diffusion properties in target cells. GL in naive form was then dissolved in ethanol in our laboratories to 10 mg ml^{-1} as stock solution and diluted with nine parts of PBS. For intranasal therapy, 50 μg or accordingly 100 μg (in 50 μl) per *in vivo* treatment was used. *In vitro* studies were performed with primary lung $CD4^+$ T cells treated with GL used at equivalent concentrations (125 ng GL per 10^6 cells ml^{-1} *in vitro* equivalent to 25 μg *in vivo* up to 500 ng ml^{-1} equivalent to 100 μg GL *in vivo* taking into account the average number of total lung cell isolated per lung) dissolved in RPMI enriched by 5% fetal calf serum.

Allergen sensitization, intra-nasal GL treatment and ovalbumin challenge in mice

Six- to eight-week-old female naive Balb/cJ mice were anesthetized with Avertine solution and subsequently treated with PBS-diluted GL in concentrations of 50 μg up to 100 μg GL per intranasal treatment for three consecutive days. Control mice received 10% ethanol (1:10 in PBS) intranasally instead. In the acute experimental asthma protocol, mice were sensitized with ovalbumin (OVA) intraperitoneally at day 0 and 14 and challenged with OVA in PBS per aerosol (10 mg OVA/ml PBS) (OVA/OVA) at days 25, 26 and 27. Two hours before each allergen challenge, mice were treated intra-nasally with GL as described above. Intranasal applications were performed as previously described (12).

At day 28, airway hyperresponsiveness (AHR) was measured by invasive plethysmography, bronchoalveolar lavage fluid (BALF) was collected and all mice were sacrificed for lung molecular analyses as previously described (19, 20). AHR data are reported as airway resistance (RL) above the baseline value. Data are expressed as mean values of RL ± SEM. Histological sections were performed from formalin-stored lungs as previously described (20, 21). T-bet-deficient mice have been previously described in a setting of experimental asthma (22). All animal experiments have been approved by the ethical committee of Rheinland Pfalz, Germany.

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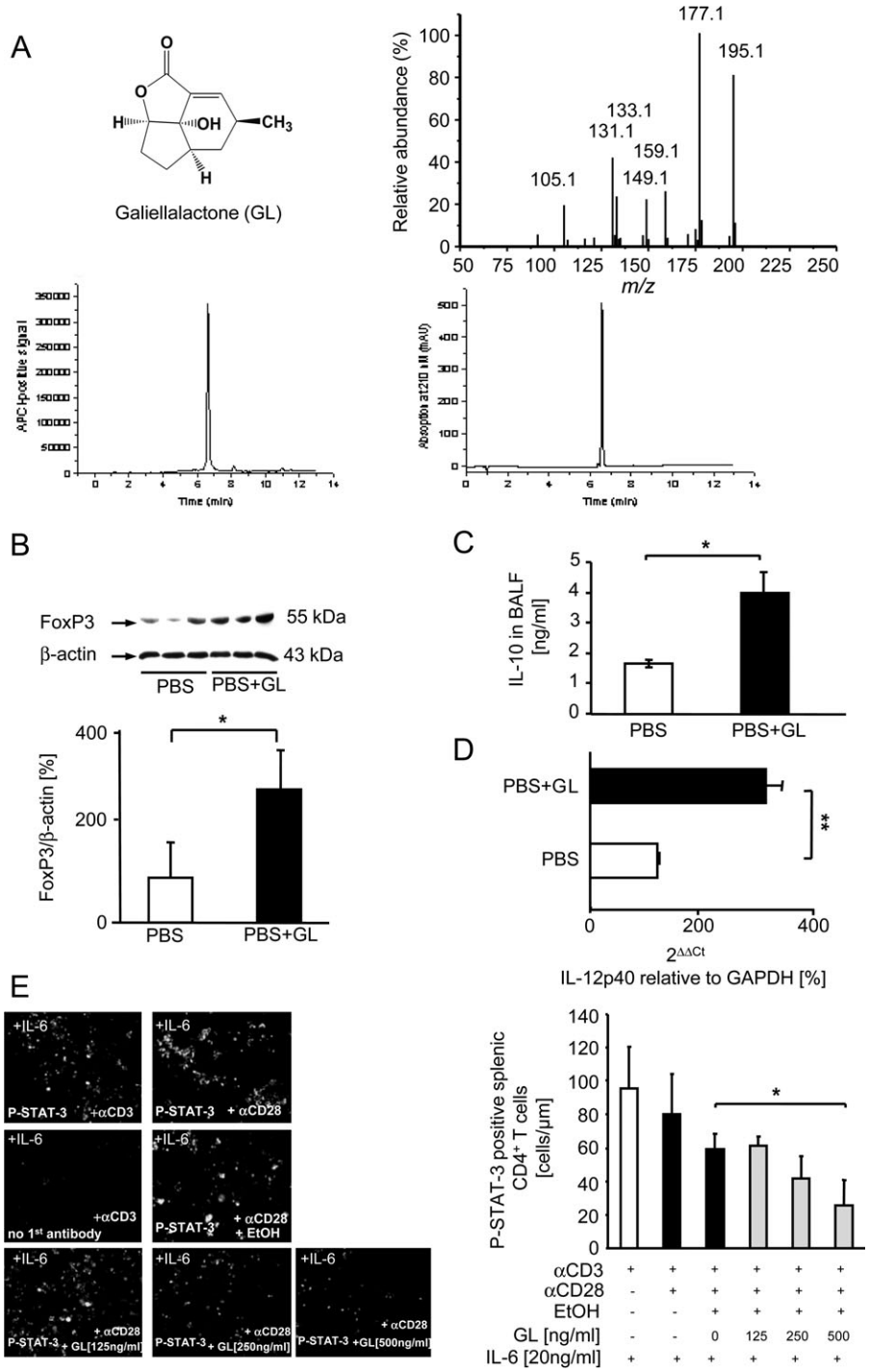

Fig. 1. GL treatment reduced pSTAT-3 in naive splenic CD4$^+$ T cells and induced lung FoxP3, IL-12p40 and IL-10 expression. (A) Upper panels: structure and mass spectrum of GL; lower panels: analysis of purified GL by UV signals at 210 nm (left-hand side panel) and detection of APCI-positive signal fragments (right-hand side panel). (B) Induction of lung FoxP3 protein expression (P = 0.023; n = 3); IL-10 release in the airways (C; P = 0.046; n = 3) and *IL-12p40* mRNA expression (D; P = 0.0023; n = 3) in the lung after GL (50 µg per intra-nasal treatment) in naive mice. (E) PSTAT-3 staining of splenic CD4$^+$ T cells pre-treated with IL-6. GL treatment reduced, in a dose-dependent manner, the number of pSTAT-3$^+$CD4$^+$ T cells that were pre-activated with IL-6 (20 ng ml^{-1}) (P = 0.032; n = 3). Data are shown as mean values ± SEM. *P < 0.05; **P < 0.01.

CD11$^+$ cells, CD4$^+$ T cells and CD4$^-$ cells isolation and culture

Murine CD11c$^+$ cells were isolated from the lung as previously described (23). Purified CD11c$^+$ cells were cultured overnight in 500 µg ml^{-1} OVA and 5% fetal calf serum in RPMI medium (2.5 × 10^{5}cells per well). Lung CD4$^+$ T cells were purified as well by using immunomagnetic separation as previously described (24). Lung CD4$^+$ T cells were

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cultured in the presence of anti-CD28 (2 μg ml^{-1}) overnight after coating for 1 h at 37°C with anti-CD3 antibodies (2.5 μg ml^{-1}) and analyzed for cytokines in their supernatants as described previously (25). Lung $CD4^-$ T cells were isolated by separating them from $CD4^+$ T cells by magnetic bead sorting. Cells were then cultured overnight in RPMI medium supplemented with 500 μg ml^{-1} OVA. Then, CD11c and CD123 expression was measured in those cells by FACS as described previously (26). Moreover, RNA was extracted for detection of indoleamine-2,3-dioxygenase (IDO) by quantitiative real-time PCR as described below.

Immunohistochemistry

Splenic $CD4^+$ T cells were incubated for 30 min with recombinant murine IL-6 (20 ng ml^{-1}) alone or with the indicated concentrations of GL in the presence of anti-CD3 (2.5 μg ml^{-1}) and soluble anti-CD28 antibodies (2 μg ml^{-1}) in duplicated wells. After that, cells were frozen for subsequent detection of pSTAT-3 expression. For immunodetection of pSTAT-3, cells were fixed in 2% paraformaldehyde in PBS for 10 min and washed twice in PBS. Cells were then permeabilized for 4 min in permeabilization buffer (0.2% Triton X-100 in PBS) and washed twice in Tris-buffered saline (TBS) buffer (0.05 M Tris hydrochloride). After 40 min of incubation in blocking buffer (3% BSA and 0.05% Tween 20 in TBS), a rabbit polyclonal anti-pSTAT-3 (Tyr705) antibody (Cell Signaling Technology, Danvers, MA, USA) was applied (1:50) in blocking buffer and incubated overnight at 4°C. Detection of positive cells was performed after cells in wells were incubated with a biotinylated goat anti-rabbit antibody solution (Vector Laboratories Inc.; 1:200 in blocking buffer) for 30 min. Finally, the Cy2–streptavidine solution (1:500 in PBS) was added, followed by incubation for 60 min at room temperature. The cells in wells were finally washed three times in PBS. Cells were then analyzed with an inverted fluorescence microscope (Zeiss) at both 200- and 400-fold magnifications.

RNA isolation and real-time PCR

Total lung tissue and purified lung cells were homogenized and RNA was then extracted by using PeQ-Gold for tissue disruption (PeQLab, Erlangen, Germany) or RNA Micro Kit (Qiagen, Hilden, Germany) for RNA preparation from isolated cells. RNA (1 μg) was used for cDNA generation using the first strand cDNA synthesis kit for reverse transcription–PCR (MBI Fermentas, St Leon-Rot, Germany). The resulting template cDNA was amplified by quantitative real-time PCR by using specific primers (see below) in 20-μl reactions in an iCycler (BioRad). For IDO and hypoxanthine phosphoribosyl transferase 1 (HPRT) quantitative PCR (5 min at 95°C and 40 cycles of 15 s at 94°C, 60 s at 60°C), an SYBR green™ (Thermo Fisher Scientific, Dreieich, Germany) technique for DNA labeling was used. For the housekeeping gene HPRT(NM_013556), the following sequences of the primers were used: 5′-GCCCCAAAATGGTTAAGGTT-3′ as forward primer and 5′-TTGCGCTCATCTTAGGCTTT-3′ as reverse primer. The primers used to detect IDO (NM-008324) had the following sequence: forward primer, 5′-AAGGGCTTCTTC-CTCGTCTC-3′ and reverse primer, 5′-AAAAACGTGTCTGG-GTCCAC-3′. FoxP3 (NM-054039) was detected with a forward primer with the sequence 5′-AGAAGCTGGGAGCTATGCA-GG-3′ and a reverse primer with the sequence 5′-GGCTAC-GATGCAGCAAGAGC-3′. For IL-12p40 (NM-008352) quantitative TaqMan-PCR studies, the following primers were used: 5′-TACTCCGGACGGTTCACGTG-3′ and 5′-GTCACTGCC-CGAGAGTCAGG-3′. All primers were designed using the primer3plus-software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and the mRNA sequences indicated.

Protein extraction and western blot analysis

Tissue proteins were extracted and protein concentration was determined as described previously (19). Mouse monoclonal antibodies against FoxP3 were purchased from e-bioscience (San Diego, CA, USA). Additional rabbit polyclonal antibodies against SOCS-3 (Upstate, Billerica, MA, USA) and goat polyclonal antibodies against β-actin were purchased from Santa Cruz Biotech (Heidelberg, Germany). Polyclonal pSTAT-6, pSTAT-5 and STAT-5 antibodies from rabbit origin were purchased from Cell Signaling. Western blot analysis was done as previously reported (21) and quantified by densitometrical analysis (BioAnalyze™; Biometra, Göttingen, Germany).

Enzyme-linked immunosorbent assay

Mouse IL-2, IL-4, IL-6, IFN-γ, IL-10 and IL-12p70 were detected using a specific sandwich ELISA (all purchased from OptEIA™; BD Pharmingen, Heidelberg, Germany). IL-17A was detected by using a DuoSet Kit (R&D Systems, Wiesbaden, Germany). Transforming growth factor (TGF)-β_1 monomers were quantified from BALF as described previously (25).

FACS analysis

The primary cells isolated from lung or spleen were stained with antibodies directed against immunosuppressive DC markers like FITC-conjugated anti-CD11c (BD Pharmingen), anti-CD123 (PE conjugated; e-Bioscience). $CD4^+$ T cells were analyzed with anti-CD4-APC (e-Bioscience), CD69-FITC and CD44-Cy7 (BD Pharmingen). Intracellular staining for FoxP3 (e-bioscience), a marker for Treg cells, was performed as described previously (27). All antibodies were diluted from 60 up to 120 ng per 100 μl in PBS per 5×10^5 total lung cells for cellular surface staining as previously standardized (26).

Statistical analysis

Values were evaluated for statistically significant differences ($P < 0.05$) by the Student's two-tailed *t*-test for independent events (Excel, PC). Data are given as mean values ± SEM. A two-way analysis of variance (ANOVA) test was used to determine the statistical significant differences of the AHR measurements among the group of mice (28).

Results

GL induces FoxP3 and IL-10 in naive lungs in vivo

GL is a known inhibitor of STAT-3 that was isolated from the ascomycete *G. rufa* after biofermentation by using chemical purification steps and HPLC techniques. GL and its mass

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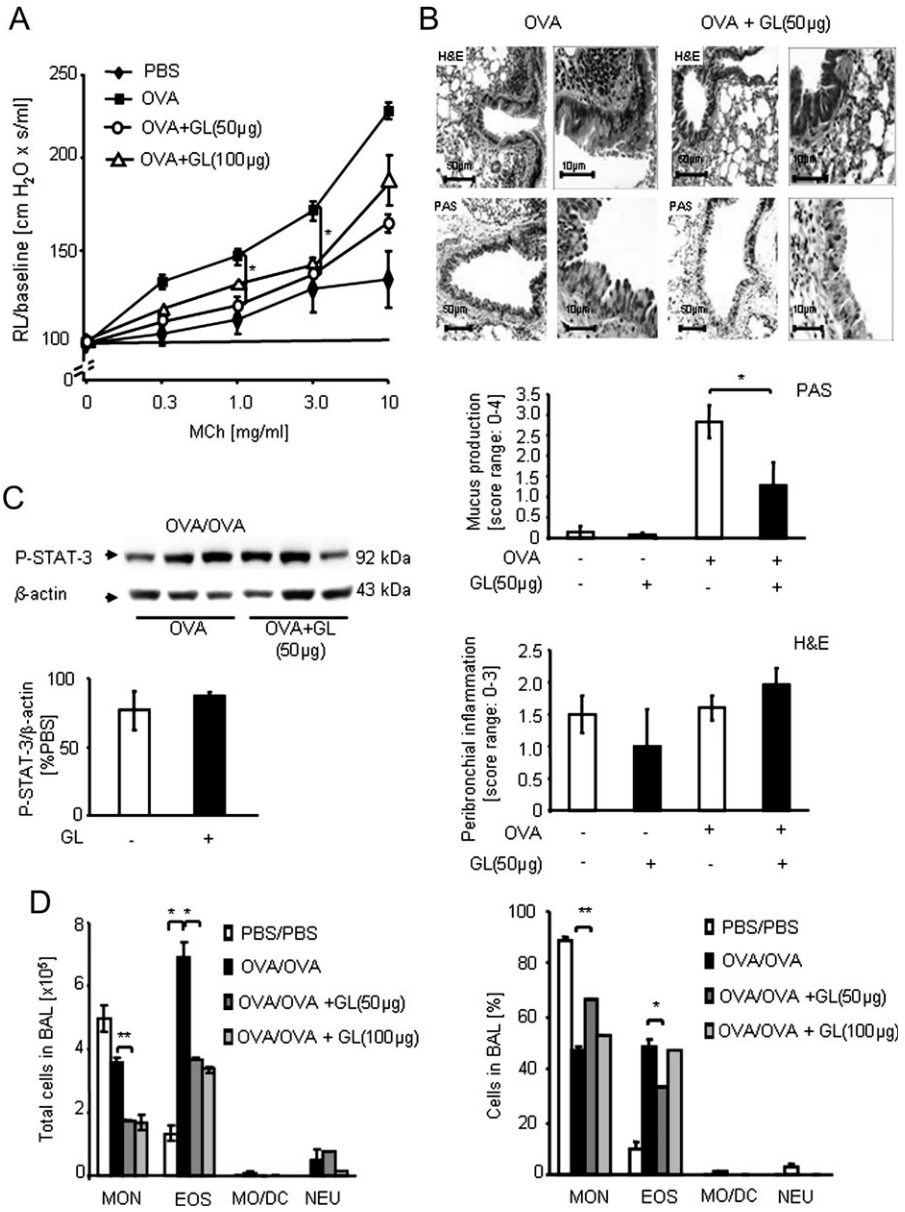

Fig. 2. Protective effect after local GL treatment in experimental murine allergic asthma before and during allergen challenge. (A) AHR was inhibited in mice treated with a moderate dose of 50 µg GL after OVA sensitization, before and during challenge compared with control mice [$P = 0.038$ (3 mg ml^{-1}), $n = 10$ and $P = 0.024$ (10 mg ml^{-1}), $n = 10$ in a student's *t*-test]. A two-way ANOVA analysis of all values was also performed [$P = 0.042$ by comparing RL/baseline values (% of baseline) of OVA untreated with OVA +GL (50 µg) treated mice]. Higher doses of GL did not significantly ameliorate lung function. (B) In at least three independent experiments of five mice per group H&E and PAS (mucus) staining were performed. In an acute model of asthma, the effect on airway inflammation after GL at a dose of 50 µg per intra-nasal treatment was analyzed. As shown, OVA-induced mucus hyper-production was significantly decreased by GL treatment ($P = 0.041$; $n = 6$) (lower panels). No significant effect was observed on the peribronchial infiltration of inflammatory cells (upper and middle panels). (C) Total lung proteins were isolated and western blot analysis was performed for pSTAT-3. The values were corrected for β-actin. No significant difference was observed between groups in pSTAT-3 expression after OVA sensitization and challenge ($P = 0.235$; $n = 3$). (D) Eosinophils ($P = 0.032$; $n = 5$) as well as mononuclear cell number ($P = 0.002$; $n = 5$) were decreased in the BALF after OVA and GL (50 µg) treatment as compared with untreated control mice. Absolute numbers (left panel) were obtained by multiplication of the values in percentage (right panel) and the total cell numbers per ml of BALF obtained after counting with a cytometer. One representative experiment out of three is shown. Data are shown as mean values ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

spectrum are shown in Fig. 1A (upper left and right panel, respectively). Analysis of purified GL by UV signals at 210 nm (Fig. 1A, lower left-hand side panel) and detection of APCI-positive signal fragments (Fig. 1A, lower right-hand side panel) are shown.

To test the effects of GL in naive lungs *in vivo*, we treated in an initial series of studies naive Balb/c mice intra-nasally with GL for three consecutive days at days 1, 2 and 3 with 50 µg in 50 µl. Twenty-four hours after the last intra-nasal GL treatment, the airway hyperresponsiveness (AHR) was

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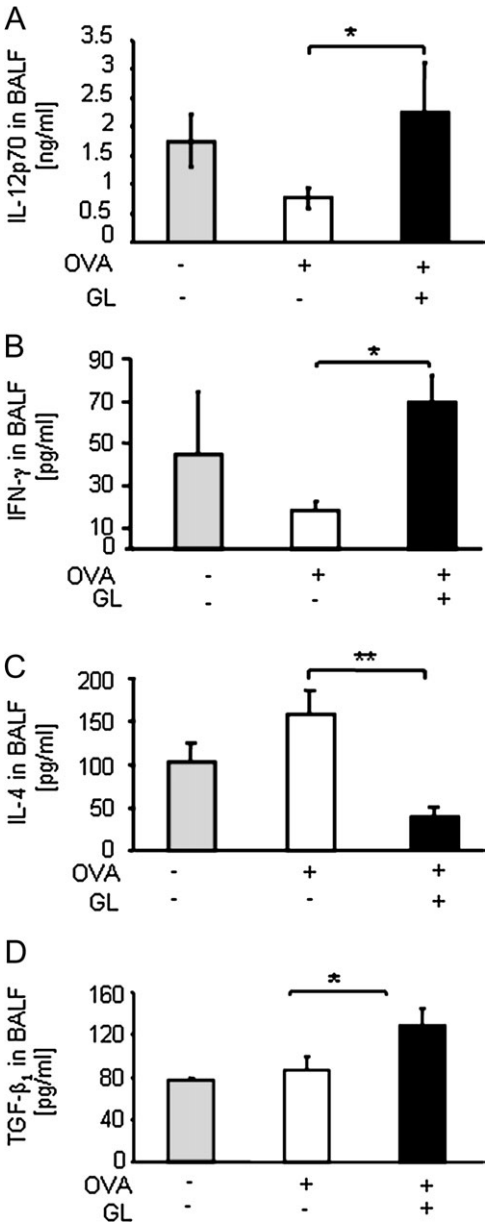

Fig. 3. GL-treated mice have increased IL-12p70 and IFN-γ and decreased IL-4 levels in their airways in a murine model of asthma. (A) IL-12p70 was found to be increased in the airways of GL (50 μg)-treated mice ($P = 0.048$; $n = 4$) along with IFN-γ, the signature T_h1 cytokine (B) ($P = 0.034$; $n = 5$). (C) IL-4 was found to be decreased in the airways of GL-treated mice as compared with untreated control mice ($P = 0.006$; $n = 6$). (D) TGF-β_1, a suppressor cytokine that induces and is also produced by Treg cells, was increased by GL treatment in a murine model of asthma ($P = 0.049$; $n = 4$). One representative experiment out of two is shown. Data are represented as mean values ± SEM. *$P < 0.05$; **$P < 0.01$.

measured and bronchoalveolar lavage was performed. Immediately after that, the mice were sacrificed and total lung protein and mRNA were isolated and analyzed using western blot and real-time PCR analysis, respectively. It was found that such a local GL treatment induced FoxP3 protein levels in the lung as compared with control untreated mice (Fig. 1B). As shown in Fig. 1C, IL-10 protein release in the bronchoalveolar lavage and *IL-12p40* mRNA (Fig. ID) in the lung were found to be increased in GL-treated mice. GL intra-nasal administration without any allergen sensitization and challenge did not influence spontaneous AHR or inflammation (data not shown), despite the fact that it induced IFN-γ release in the BALF and in the lung $CD4^+$ T-cell supernatants under co-stimulatory conditions (Supplementary Figure SI is available at *International Immunology* Online). Thus, local treatment with GL may be used as a strategy to induce T_h1 and immunosuppressive responses before antigen exposure without modulating basal levels of AHR and lung inflammation.

Since IL-6 signaling is important for Treg cells and T_h17 development, we then looked for specific IL-6 signaling. To this aim, we analyzed pSTAT-3 expression after GL treatment *in vitro* in isolated naive spleen $CD4^+$ T cells. It is known that STAT-3 binding to target promoter is inhibited by GL in HepG2 hepatocytes (14) and Supplementary Figure SII (available at *International Immunology* Online). As shown in Fig. 1E, GL inhibited the number of $CD4^+$pSTAT-3^+ T cells after immunohistological staining of IL-6-treated spleen $CD4^+$ T cells. Thus, GL selectively inhibits IL-6/STAT-3 signaling in $CD4^+$ T cells in a dose–response manner (Fig. 1E, lower panels and as quantified in a graph on the right-hand side).

Protective effect of galiellalactone therapy on allergen-induced AHR and pathology

In subsequent studies on the therapeutic use of GL in OVA-sensitized mice, we found that mice treated with 50 μg but not with 100 μg of GL for three consecutive days immediately before as well as during allergen challenge showed significantly ameliorated methacholine-induced airway hyperresponsiveness (AHR) compared with control mice (Fig. 2A). Moreover, GL (50 μg) treatment led to a selective decrease in airway mucus production without significantly changing other classical airways inflammatory parameters as compared with untreated control mice (Fig. 2B). Under these experimental conditions, no significant change in total lung pSTAT-3 was observed (Fig. 2C). Moreover, the number of infiltrating eosinophils was also found to be reduced in the BALF of GL-treated mice as compared with that of untreated mice (Fig. 2D). Taken together, these data demonstrated a marked therapeutic effect of GL at a dose of 50 μg in a murine model of OVA-induced allergic asthma.

GL intra-nasal treatment induced T_h1 and TGF-β_1 and decreased IL-4 in the airways of antigen-sensitized and challenged mice

GL is known to inhibit pSTAT-3 DNA binding (14) and STAT-3 phosphorylation in lung $CD4^+$ T cells (Fig. 1E) and therefore might target several cytokine and transcription factor promoter activities and gene expression, e.g. IL-4, IL-6, IL-10 and accessory signaling molecules. For this reason, we analyzed T_h1-inducing cytokines upon GL treatment in experimental asthma. We observed that IL-12p40 mRNA was up-regulated in the airways of GL-treated mice compared with control, untreated naive mice (Fig. 1D). However, IL-12p40 is a part of both IL-23 and IL-12p70. Since

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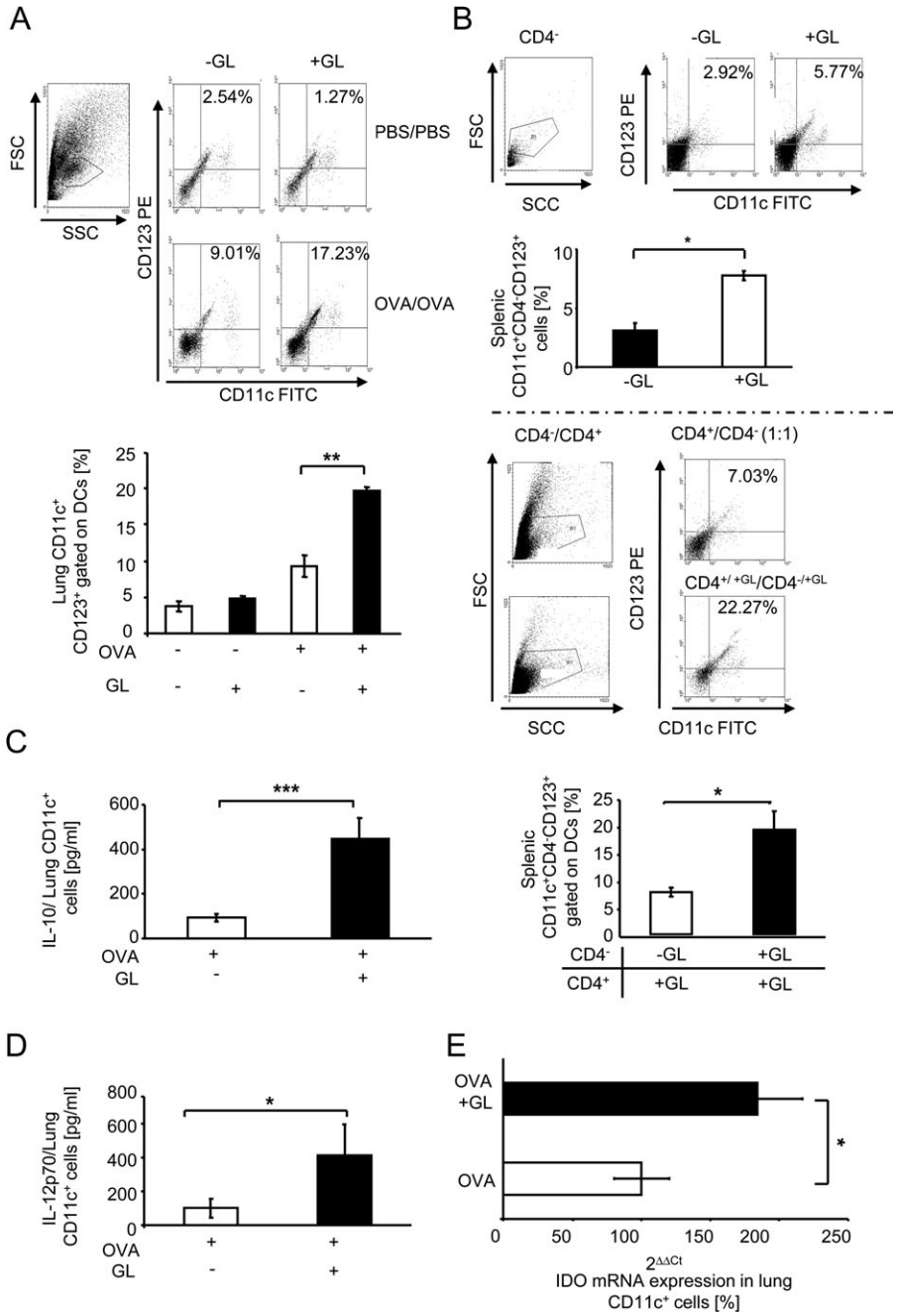

Fig. 4. Lung and splenic CD123^{hi} CD11c^{+} cells released more IL-10 and IL-12p70 in the airways after GL treatment in a murine model of allergic asthma. (A) Increased number of lung CD11c^{+} cells express CD123 (IL-3 receptor α) on their surface if isolated from GL (50 μg)-treated mice compared with those isolated from untreated control littermates after OVA sensitization and challenge ($P = 0.004$, $n = 4$). (B) CD4^{-} splenocytes were cultured *in vitro* without and with GL (250 ng ml^{-1}) (upper panels) and afterward co-cultured with GL-pre-treated splenic CD4^{+} T cells (lower panels). FACS analysis was performed after staining for CD11c and CD123. Splenic CD4^{-} cells treated *in vitro* with GL expressed increased number of CD11c^{+}CD123^{+} as compared with untreated cells (upper panels). Additionally, GL-pre-treated splenic CD4^{+} T cells further induced the CD11c^{+}CD123^{+} cell population in GL-treated splenic CD4^{-} cells ($P = 0.008$; $n = 5$). (C and D) CD11c^{+} cells isolated from the lungs of GL (50 μg)-treated mice released increased IL-10 (C) and IL-12p70 (D) compared with those isolated from untreated control mice ($P < 0.001$; $n = 3$ and $P = 0.039$; $n = 4$, respectively). (E) Lung CD11c^{+} cells express increased amounts of indoleamine-2,3-dioxygenase (IDO) mRNA after GL (50 μg) treatment *in vivo* and after OVA recall (500 μg ml^{-1}) *in vitro* ($P = 0.047$; $n = 3$). One representative experiment out of two is shown. Data are represented as mean values ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

IL-12p70 induces T_h1, whereas IL-23 is known to support the development of the pro-inflammatory T_h17 cells, we then looked at IL-12p70 protein production by ELISA in a murine model of asthma after OVA sensitization and challenge in untreated and GL-treated mice (Fig. 3A). As shown, GL induced IL-12p70 (p35/p40 heterodimer)

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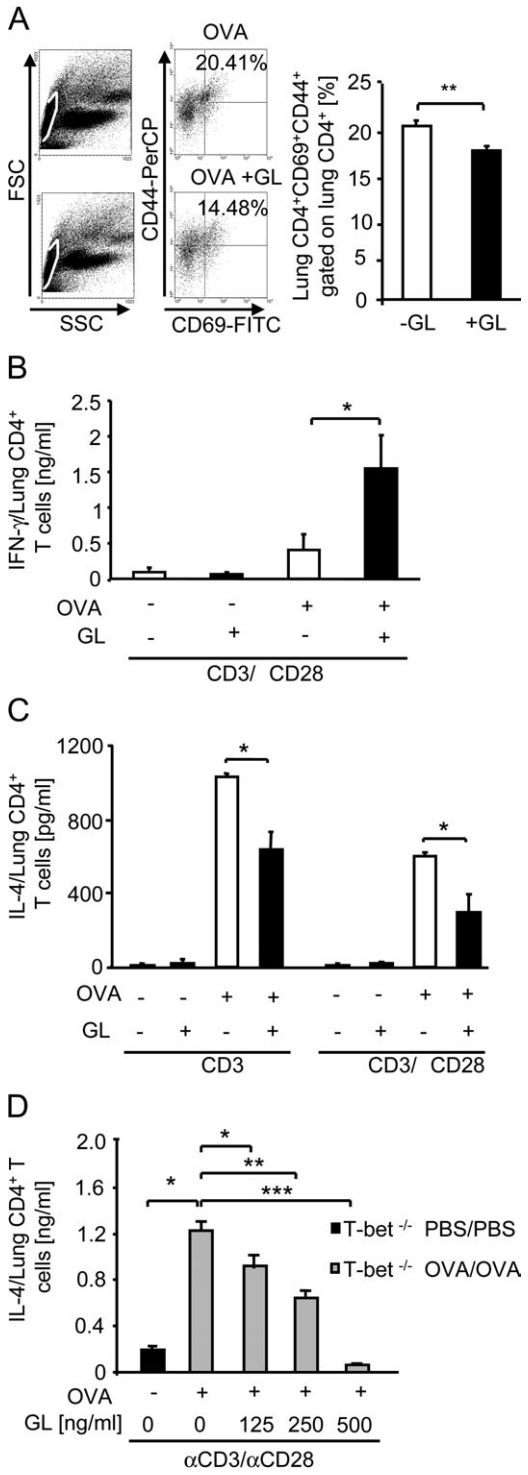

Fig. 5. GL inhibited IL-4 release by T-bet$^{(-/-)}$ lung CD4$^+$ T cells isolated from OVA-sensitized and -challenged mice. (A) Lung CD4$^+$ T cells isolated from the lungs of GL (50 μg)-treated mice contain significantly lower number of CD44- and CD69-positive cells, indicating decreased activation of lung CD4$^+$ T effector cells ($P = 0.005$; $n = 4$) upon GL treatment. (B–C) Under co-stimulatory conditions, lung CD4$^+$ T cells isolated from GL-treated mice released increased amounts of IFN-γ (B), whereas IL-4 (C) was down-regulated in these cell supernatants as compared with those obtained from lung CD4$^+$ T cells isolated from untreated mice. Supernatants were measured by ELISA after overnight culture ($P = 0.03$, $n = 4$ and $P = 0.025$, $n = 4$, respectively). (D) In T-bet-deficient lung CD4$^+$ T cells, IL-4 was suppressed in a dose-dependent manner by GL *in vitro* [$P = 0.021$, $n = 2$ for GL (125 ng ml^{-1}); $P = 0.006$, $n = 3$ for GL (250 ng ml^{-1}) and $P < 0.001$, $n = 2$ for GL (500 ng ml^{-1})]. One representative experiment out of three is shown. Data are represented as mean values ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

when applied intra-nasally before and during allergen challenge as well as the mRNA expression of p40 in naive mice (see Fig. 1D). Consistent with the increase of IL-12p70 in the BALF, we found up-regulation of the signature cytokine of T_h1 cells, IFN-γ (Fig. 3B), and a decrease of the T_h2 cytokine IL-4 (Fig. 3C) in the airways of OVA-sensitized and -challenged GL-treated mice compared with the OVA/OVA untreated control mice. Therefore, GL therapy might prevent AHR and airway eosinophilia by inducing a T_h1 phenotype as well as by inhibiting T_h2 cells locally. The pro-inflammatory effect of GL at higher doses is at the moment not well understood but it could be that high levels of IFN-γ in the airways after application of higher doses of GL might negatively influence inflammation and other parameters in experimental asthma.

Furthermore, TGF-β_1, a cytokine with the capacity to stimulate the differentiation of T_h17 as well as Treg cells, was found to be increased in the airway of GL-treated mice compared with the control untreated mice after allergen challenge (Fig. 3D).

Increased IL-10 and IL-12p70 release by lung DC isolated from GL-treated mice

We next asked whether the change in cytokine production in the BALF would reflect changes in local lung DCs' character. To this aim, we analyzed the number of cells co-expressing CD11c and CD123 (IL-3-receptor-α), a marker of immunosuppressive myeloid DCs. It was found that they were increased after GL therapy (Fig. 4A). To demonstrate a direct effect of GL on DCs, we isolated splenic CD4-negative cells (CD4$^-$), treated them with GL (+GL; 250 ng GL per ml RPMI, 5% FCS) or left them untreated (−GL) and 24 h later analyzed the expression of CD123 and CD11c. As shown in Fig. 4A, GL induced the expression the IL-3 receptor α chain (CD123) on those cells. Moreover, when CD4$^-$ cells were incubated overnight with 250 ng ml^{-1} GL (+GL) and then co-cultured again overnight with an equal number of GL-treated (250 ng ml^{-1}) CD4$^+$ T cells (+GL), an induction of CD11c$^+$CD123$^+$ cells mediated by the GL-primed CD4$^+$ T cells was found (Fig. 4B, lower panel). Therefore, we next focused on cytokine production of isolated lung CD11c$^+$ cells after GL treatment. Increased protein levels of IL-10 and IL-12p70 were measured in the supernatants of lung CD11c$^+$ cells isolated from GL-treated mice compared with those isolated from untreated control mice (Fig. 4C and D, respectively). These findings indicate an expansion of the DC-1 population that released enhanced levels of IL-10 and IL-12 in the lung after GL treatment. Thus, GL treatment might prevent experimental asthma because it affects CD11c$^+$ cell cytokine production (IL-10) (17) and induces their ability to prime CD4$^+$ T cells to develop into Treg cells. Additionally, we found increased

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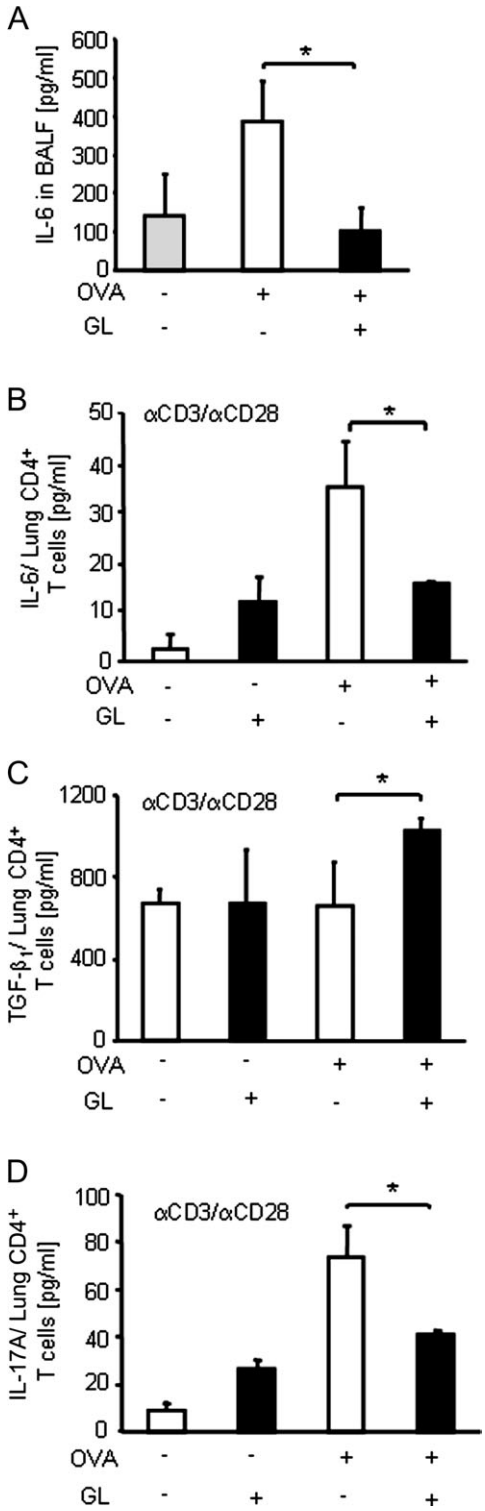

Fig. 6. Down-regulation of T_h17 cells in the lung of GL-treated mice compared with control untreated mice in a murine model of allergic asthma. GL (50 μg)-treated mice released decreased levels of IL-6 (A) in the airways and in lung $CD4^+$ T-cell supernatants (B) as compared with OVA-sensitized and untreated control mice ($P = 0.049$; $n = 3$). (C) Lung $CD4^+$ T cells from *in vivo* GL-treated mice released increased amounts of TGF-β_1 after OVA sensitization and challenge ($P = 0.049$; $n = 3$). (D) In addition, a decreased IL-17A level was found, probably as a result of GL-dependent decrease of IL-6 release by $CD4^+$ T cells was observed ($P = 0.045$; $n = 3$). One representative experiment out of two is shown. Data are represented as mean values ± SEM. $^*P < 0.05$.

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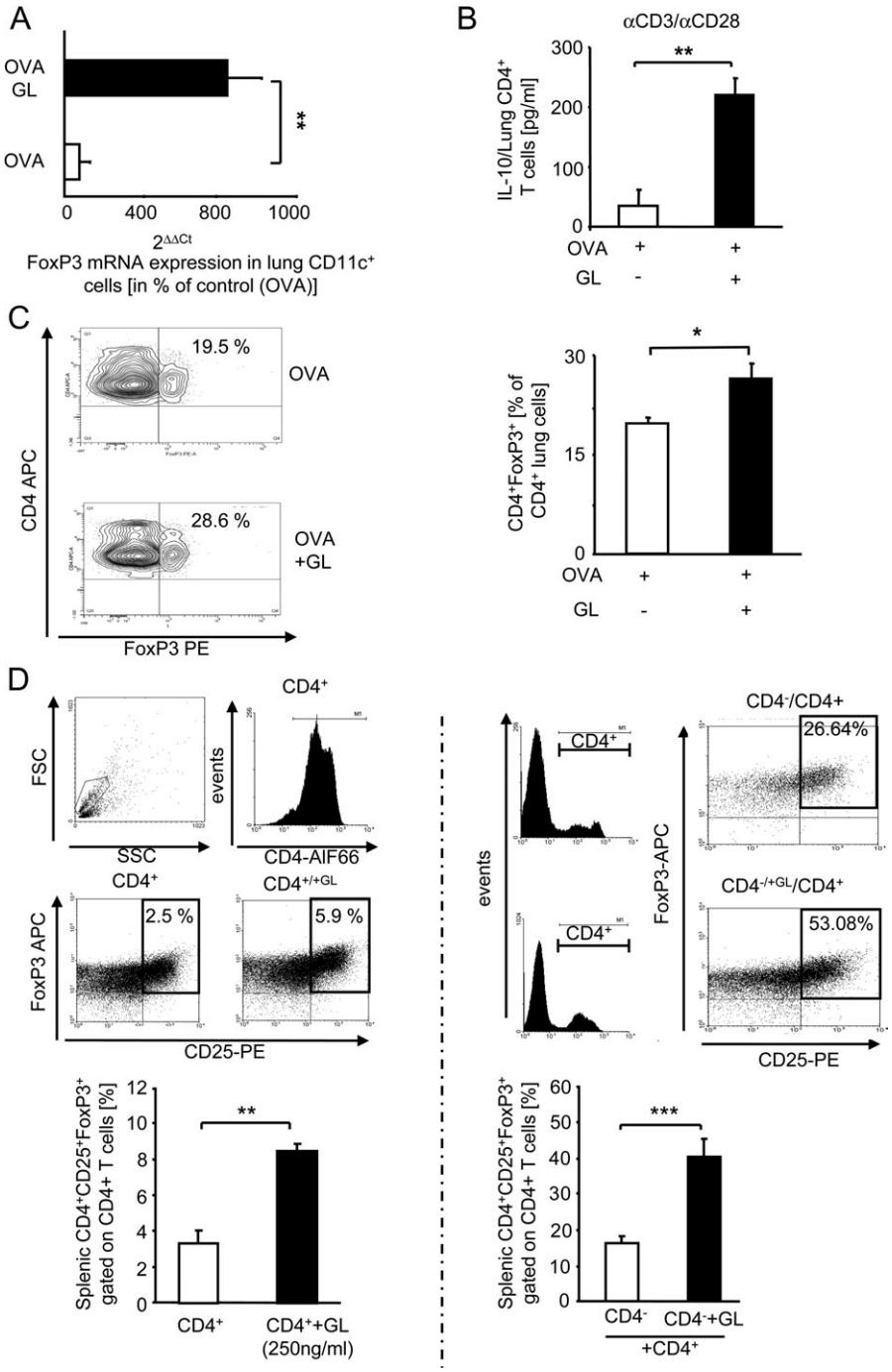

Fig. 7. GL-treated splenic CD4$^-$ cells enhanced FoxP3 and IL-10 expression in splenic untreated CD4$^+$ T cells. Since TGF-β_1 is known to induce FoxP3, we then analyzed the expression of FoxP3 in lung CD4$^+$ T cells in GL (50 μg)-treated and control mice in a murine model of allergic asthma. (A) Consistent with the findings in naive mice, lung FoxP3 mRNA was increased in GL (50 μg)-treated mice compared with the control littermates after OVA sensitization and OVA challenge ($P = 0.0013$; $n = 6$, respectively). (B) Lung CD4$^+$ T cells also released increased amounts of IL-10 indicating the presence of Treg cells after local GL treatment ($P = 0.005$; $n = 3$). (C) OVA-sensitized and -challenged mice treated intranasally with 50 μg GL before and during allergen challenge showed increased numbers of lung CD4$^+$ T cells expressing FoxP3 after FACS intracellular analysis ($P = 0.019$; $n = 4$). (D) FoxP3 expression was induced in GL-treated splenic CD4$^+$ T cells ($P = 0.021$; $n = 4$; left-hand side lower panels). FoxP3 expression in untreated CD4$^+$ T cells was also significantly increased after co-culture (1:1) with splenic CD4$^-$ cells pre-incubated for 24 h with GL (250 ng ml^{-1}) compared with untreated splenic CD4$^-$ cell co-culture ($P < 0.001$, $n = 4$; -right-hand side panels). One representative experiment out of three is shown. Data are represented as mean values ± SEM. *$P < 0.05$; **$P < 0.01$.

mRNA expression of indoleamine-2,3-dioxygenase (IDO) in lung CD11c$^+$ cells treated with OVA and GL (Fig. 4E). Plasmacytoid DCs expressing IDO are known to be potent inducers of differentiation of Treg cells and therefore are able to maintain an immunosuppressive tolerance in the lung (29).

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GL inhibited IL-4 release by lung CD4$^+$ T cells isolated from T-bet-deficient mice

We next isolated CD4$^+$ T cells from the lung of GL-treated and untreated mice after OVA sensitization and challenge and measured their activation state (Fig. 5A). As shown, the CD4$^+$ T cells isolated from the lung of OVA-sensitized and -challenged (OVA/OVA) and GL-treated mice have a less activated phenotype compared with those isolated from untreated mice. Moreover, CD4$^+$ T cells isolated from the lung of GL-treated mice displayed a T_h1-like phenotype rather than a T_h2-like phenotype as demonstrated by increased IFN-γ (Fig. 5B) and decreased IL-4 (Fig. 5C) levels in their supernatants compared with lung CD4$^+$ T cells isolated from untreated and OVA-sensitized and -challenged and untreated mice. Then, we exposed primary lung CD4$^+$ T cells from T-bet$^{-/-}$ OVA/OVA-treated mice to increasing doses of GL and measured IL-4 production in the supernatant. As shown in Fig. 5D, GL inhibited the IL-4 production in the supernatants of T-bet-deficient lung CD4$^+$ T cells, indicating that GL inhibited IL-4 via a T-bet-independent mechanism. In addition, we noticed that the decrease of IL-4 in the presence of T-bet after *in vitro* treatment with GL did not follow the classical linear dose-dependent decrease indicating an interfering effect of T-bet on GL-mediated IL-4 inhibition (data not shown). In contrast, in the absence of T-bet, GL-mediated IL-4 suppression followed a dose–response curve, indicating that T-bet might interact with pSTAT-3 on the IL-4 promoter.

GL inhibited IL-6 signaling and decreased the IL-17 production in the lung

Treg cells have been described as the opposite developmental pathway of a newly described subset of pro-inflammatory CD4$^+$ T cells known as T_h17 because of releasing IL-17. We then started to analyze T_h17 cells in the lung of GL-treated mice. TGF-β_1 together with IL-10 reinforces Treg cell development (30, 31) or in synergy with IL-6 it induces T_h17 cells (32). In accordance to this concept, we found significantly decreased levels of IL-6 (Fig. 6A) in the BALF of GL-treated mice. Furthermore, CD4$^+$ T cells isolated from lungs of GL (50 μg)-treated mice released significantly less IL-6 (Fig. 6B) and IL-17A (Fig. 6D) but increased levels of TGF-β_1 (Fig. 6C) as compared with those isolated from control untreated mice.

GL mediated the induction of lung FoxP3 and IL-10-producing CD4$^+$ T cells in vivo

We thus reasoned that the decreased activation state of lung CD4$^+$ T cells and the decreased number of T_h17 cells (33) after GL (50 μg) treatment could be accompanied by an increased number of Treg cells as we found that GL led to increased FoxP3 expression in the lungs of naive mice (Fig. 1B). TGF-β_1 is known to induce FoxP3 and accordingly, TGF-β_1 induction was accompanied by an increased amount of Foxp3 (Fig. 7A) and of CD4$^+$FoxP3$^+$ T cells in the lung of GL-treated mice (Fig. 7C). Furthermore, lung CD4$^+$ T cells isolated from GL-treated mice released five times as much IL-10 as those isolated from untreated mice (Fig. 7B). *In vitro* treated spleen CD4$^+$ T cells also exhibited an enhanced Treg cell phenotype (Fig. 7D, left panels) but this difference although statistically significant was not that impressive. To further investigate whether CD4$^+$ T cells would become Tregs (Foxp3$^+$) because they are exposed to GL-treated DCs, we co-incubated untreated splenic CD4$^+$ T cells with CD4$^-$ cells pre-treated with 250 μg ml^{-1} GL. This resulted in a 2-fold increase of FoxP3 expression in CD4$^+$ T cells (Fig. 7D, lower right panels). Taken together, these data show an induction of Treg cells dependent on DCs upon GL treatment (17, 34).

Reduction of SOCS-3 and IL-2 signaling after GL treatment

STAT-5 has been recently shown to inhibit IL-12-mediated T_h1 cell development via SOCS-3 and STAT-3, resulting in T_h2 cell development (35). We next analyzed SOCS-3 in the lung of untreated and GL-treated mice. GL is known to decrease STAT-3 binding to target promoter, thus reduced SOCS-3 transcriptional activation would reflect the lack of STAT-3 binding to the target promoter. As shown in Fig. 8A, *in vivo* treatment with GL resulted in decreased SOCS-3. Consistently, CD2-specific STAT-3-deficient mice show decreased SOCS-3 expression in the lung, a signaling molecule inducing T_h2 and IL-17-producing cells (Fig. 8B) (36). We next investigated whether pSTAT-5 was also decreased after GL treatment. As shown in Fig. 8C, p-STAT-5 was decreased in the lung of GL-treated mice as compared with untreated mice. Decreased pSTAT-5 might reflect decreased IL-2. We thus next analyzed IL-2 levels in the presence or absence of T-bet in the supernatants of lung CD4$^+$/CD4$^-$ T cells isolated from OVA-sensitized and -challenged wild-type and T-bet-deficient mice. As shown, GL-treated T-bet$^{-/-}$ CD4$^+$ T cells showed a significant decrease of IL-2 levels in their supernatants (Fig. 8D). Since low levels of IL-2 are known to induce Treg cells, we conclude that also in the absence of T-bet, GL could be used as an inducer of Treg cells (17, 37).

Discussion

Allergic asthma is a disease induced by allergens like pollen or house dust mite exposition in predisposed subjects (38). Asthmatic patients are commonly treated with glucocorticoids (39) and to date the only alternative treatment is restricted to allergen-mediated immunotherapy (40). Based on the side-effects of glucocorticoid treatment such as generalized immune suppression, diabetes and osteoporosis (41–43), we have focused on the new fungal compound GL, a fungal metabolite from the ascomycete *G. rufa* (13). We could show that GL inhibited STAT-3 signaling and AHR by molecular mechanisms involving down-regulation of pSTAT-5 resulting in CD4$^+$Foxp3$^+$ Treg cells induction in treated mice. In fact, GL inhibited pSTAT-5 resulting in reduced IL-2 production and IL-2 signaling in lung CD4$^+$ T cells independently from T-bet. Furthermore, we describe here that GL induced CD11c$^+$CD123$^+$ cells, thus creating an anergic environment in which Treg cells (CD4$^+$FoxP3$^+$) can develop and then may inhibit effective immunoresponses against the allergen. We also found that this effect is mediated via inhibition of STAT-3 binding to its responsive elements on the DNA resulting in SOCS-3 inhibition, which is down-regulated in the absence of STAT-3 in T cells.

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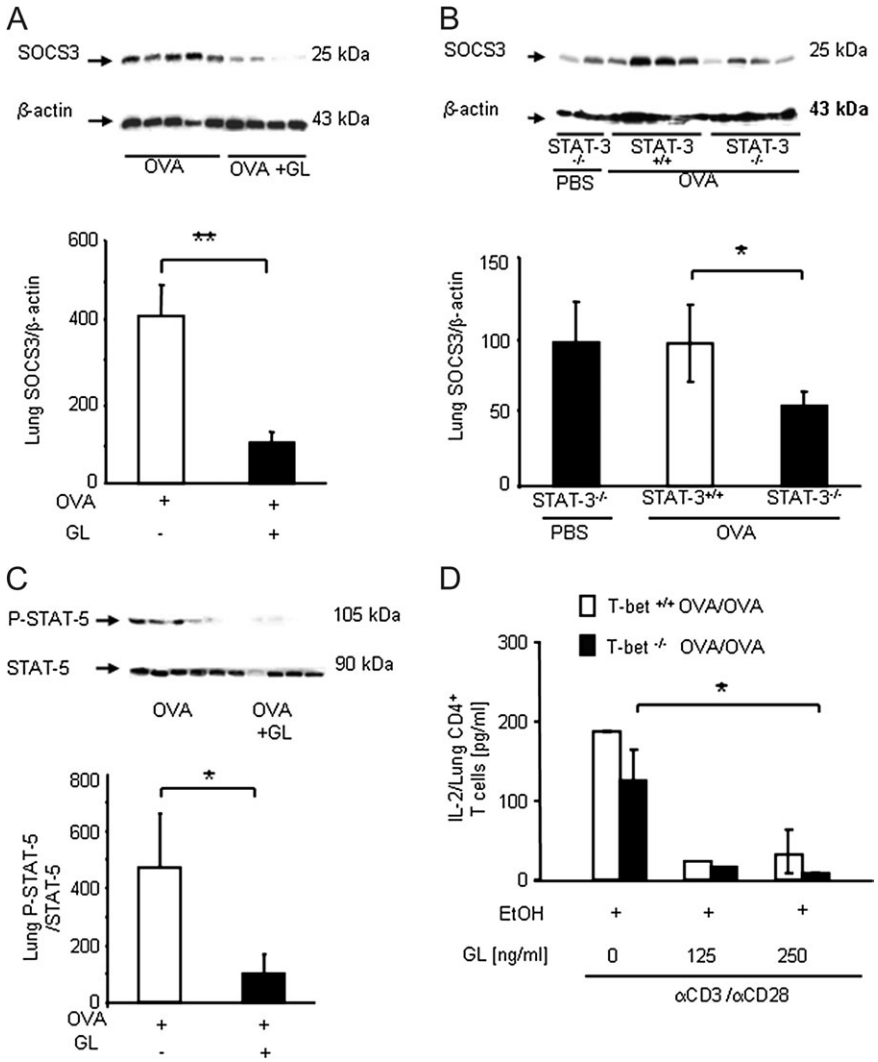

Fig. 8. GL treatment decreased IL-2 signal transduction and SOCS-3 in the lung in a murine model of asthma. (A) STAT-3 dysfunction after intra-nasal GL (50 μg) therapy in mice reduced SOCS-3, which is known to induce T_h2 responses in allergic diseases ($P = 0.0029$; $n = 5$). (B) Genetic depletion of STAT-3 in T cells decreased SOCS-3 expression in lung tissue in a murine model of asthma ($P = 0.045$; $n = 3$). STAT-3flox/flox mice were obtained from S. Akira and K. Takeda (Osaka University) and were described previously (18). (C) pSTAT-5 levels also were down-regulated after GL (50 μg) *in vivo* in an acute model of allergic asthma ($P = 0.041$; $n = 5$). One representative experiment out of three is shown. (D) IL-2 was decreased in the supernatants of lung $CD4^+$ T cells treated with increasing doses of GL *in vitro* especially in T-bet-deficient $CD4^+$ T cells ($P = 0.047$; $n = 3$). Data are represented as mean values ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

Thus, down-regulation of pSTAT-5 and SOCS-3 resulted in induction of Treg cells and inhibition of T_h17 cells, thus ameliorating experimental asthma.

The secondary fungal metabolite GL inhibited IL-6 signaling pathways by blocking the binding of STAT-3 on several cytokine promoters, leading to dysregulated cytokine release by different cell types as demonstrated for palindromic activation sites inducing IFN-γ production (44) and missing activation of signal molecules (15). In naive mice, it was found that GL (50 μg) treatment enhanced IFN-γ and FoxP3 levels in the airways without affecting basal airway hyperresponsiveness (AHR) and peribronchial inflammation. Similarly, intra-nasal application of GL (50 μg) after allergen sensitization and before and during the OVA challenge phase reduced AHR and goblet cell metaplasia in the lung as characteristic features of experimental asthma. The immunological findings accompanying this amelioration of experimental asthma are the induction of lung DCs producing more IL-10 and IL-12p70, the expansion of regulatory $CD4^+$ T cells co-expressing FoxP3 and a decrease of the T_h17 cells in the airways of GL-treated mice.

IL-6 is a pro-inflammatory cytokine produced by DCs, mast cells and B cells known to induce T_h2 cell differentiation while inhibiting Treg cells (45, 46). Consistently, we previously demonstrated that local treatment with αIL-6R antibodies before and during allergen challenge decreased the presence of activated STAT-3 in the lung of αIL-6R-treated mice (47) as compared with untreated mice. This molecular effect was accompanied by decreased T_h2 via apoptosis and increased Treg cells in the lung of treated mice (12).

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Because GL induced IL-12p70 and IFN-γ and decreased the IL-6 in the airways, it could be considered as a novel therapeutic approach for experimental allergic asthma because it recapitulated the effects of αIL-6R antibodies treatment *in vivo*. Moreover, we demonstrated that the targets of GL besides $CD4^+$ T cells are DCs, which after treatment with this pSTAT-3 and pSTAT-5 inhibitor produced more IL-12p70 and IL-10. These latter are predominant features in directing a protective local T_h1 immune response and reducing T_h2 and T_h17 cytokine production (48, 49). While αIL-4 antibody treatment is known to suppress lung inflammation and AHR in experimental asthma (21), endogenous IL-17 is essential during antigen sensitization to establish allergic asthma (48). Moreover, IL-17 has been shown to be regulated in an IL-4-dependent manner since mice deficient in IL-4Rα showed a marked decrease in IL-17 concentration with decreased eosinophil recruitment (50). More importantly, IL-17 deficiency seems to prevent development of allergic immune responses in the human lung mediated by IL-4 (51, 52). GL (50 μg) treatment reduced the production of the T_h2 cytokine IL-4 but also IL-17A (see Fig. 6D).

We thus reasoned that increased levels of IL-17-producing $CD4^+$ T cells in allergic disease may be secondary to decreased T_h1 responses resulting in worsening of allergic lung pathology (53, 54). To address this point, we analyzed IL-17A production after GL treatment in the absence of T-bet in lung $CD4^+$ T-cell culture. We found, as shown for IL-2 and IL-4 in this paper, that IL-17A is also inhibited in a dose–response manner in the absence of T-bet by GL (Supplementary Figure SIII is available at *International Immunology* Online). Finally, we demonstrated that the inhibition of the T_h2 component and the induction of the T_h1 cells in the lung of GL-treated mice did not follow a classical dose-dependent inhibition in the presence of T-bet. When GL was used in T-bet-deficient $CD4^+$ T cells, a dose response effect on IL-2, IL-4 and IL-17 inhibition was observed, indicating that T-bet interferes with pSTAT-3 on the promoter of IL-2, IL-4 and IL-17 genes. This observation will need further investigations.

Both, Treg cells development and T_h17 inhibition were proven to be linked to the inhibition of STAT-3–DNA binding, which resulted in expansion of tolerogenic DC and Treg cell differentiation while inhibiting T_h17 pathways (55).

The expression of SOCS-3, a well-known target gene of STAT-3 (56), regulates onset and maintenance of T_h2-mediated allergic responses and was reduced by this fungal compound and led to a decrease of T_h2 T-cell development (15). The decrease of SOCS-3 upon GL treatment is consistent with the increase in $CD4^+Foxp3^+$ Treg cell number in GL-treated mice. Consistently, it has been reported that SOCS-3 inhibits Treg cell functions and it is down-regulated in Treg cells (57). Consistent with our results, it has been shown that SOCS-3-deficient $CD4^+$ T cells produced more TGF-β and IL-10 but less IL-4 than control T cells (36).

Our data are consistent with a role of STAT-3 described by Radojcic *et al.* (58) in graft versus host disease (GVHD) in a murine model. In this study, it was demonstrated that STAT-3 signaling in donor mature T cells provides a direct link between alloreactive T-cell-mediated tissue damage and favorable Treg reconstitution post-transplant. Moreover, in this model, STAT-3 ablation in alloreactive T cells limits their *in vivo* proliferation and expansion in the secondary lymphoid tissues via expansion of the Treg cells. A high T-cell proliferative rate is known to correlate with acute GVHD, and the control of alloreactive T-cell proliferation is the aim of many therapies used for GVHD prophylaxis after allogenic bone marrow transplantation. However, these data are different from those reported by Pallandre *et al.* (59) and an explanation for that could be that GL targets different cell types as those achieved with small interfering RNA strategies. In fact, we demonstrated in this paper that GL targeted preferentially DCs and T cells.

Moreover, consistent with our results, in tumor setting, inactivation of STAT-3 in hematopoietic cells results in the repression of IL-23 production by tumor-associated macrophages and DCs and up-regulation of IL-12 production in DCs. Surprisingly, but consistent with the immune system associated with the tumor, in that model an anti-tumor effect of STAT-3 inhibition was in part due to decreased activity of $FoxP3^+$ Treg cells (60, 61).

In an allergic asthma model we found that inhibition of STAT-3 results in down-regulation of the IL-23 and IL-2 pathways, as confirmed by the down-regulation of STAT-5 that results in up-regulation of Treg cells and down-regulation of T_h17 cells.

In conclusion, the inhibition of the STAT-3/STAT-5/SOCS-3-dependent feedback loop after therapy in mice is an important step toward a local therapy for allergic diseases including an inhibition of the T_h2 development combined with an up-regulation of the Treg cells/T_h1 differentiation while suppressing IL-17.

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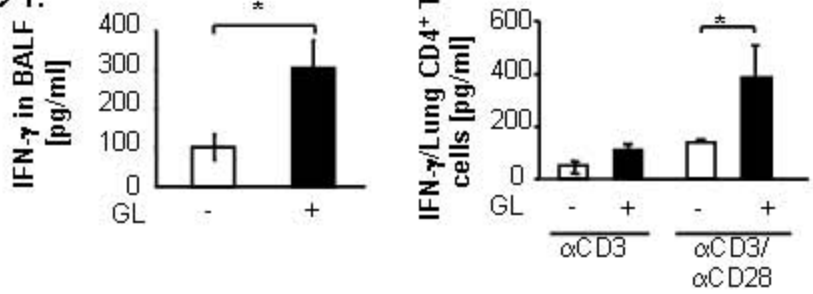
Supplementary Figures

S I.



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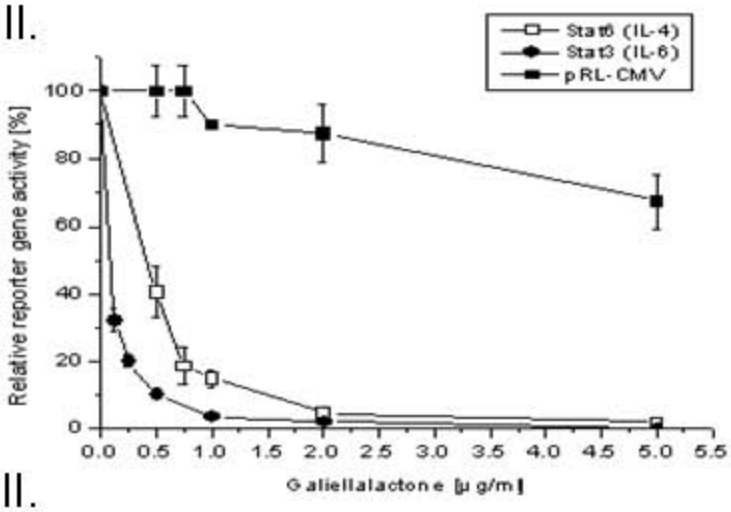

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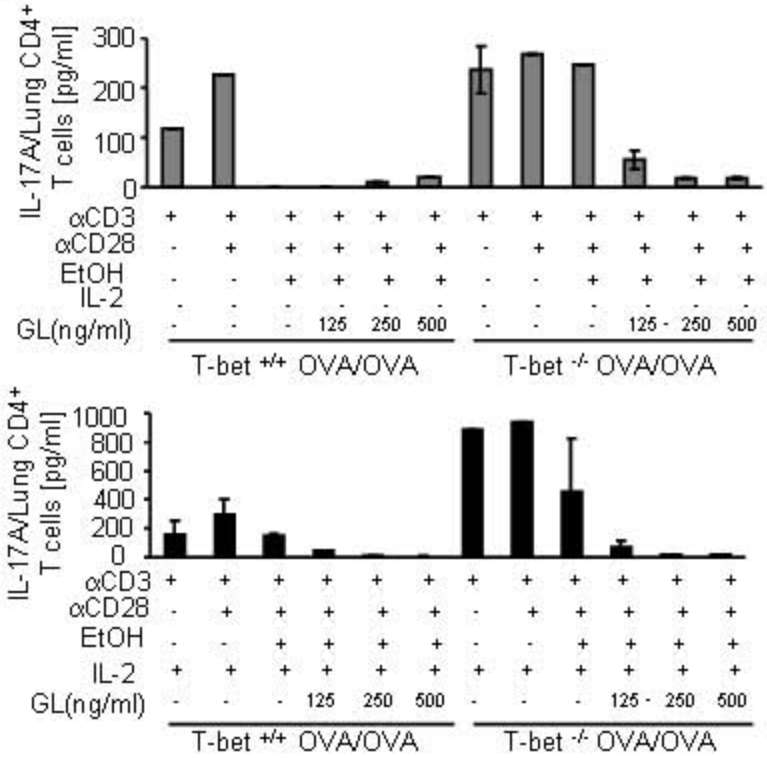

S IV.