Role of Tyk-2 in Th9 and Th17 cells in allergic asthma

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In a murine model of allergic asthma, we found that Tyk-2(-/-) asthmatic mice have induced peribronchial collagen deposition, mucosal type mast cells in the lung, IRF4 and hyperproliferative lung Th2 CD4+ effector T cells over-expressing IL-3, IL-4, IL-5, IL-10 and IL-13. We also observed increased Th9 cells expressing IL-9 and IL-10 as well as T helper cells expressing IL-6, IL-10 and IL-21 with a defect in IL-17A and IL-17F production. This T helper phenotype was accompanied by increased SOCS3 in the lung of Tyk-2 deficient asthmatic mice. Finally, in vivo treatment with rIL-17A inhibited local CD4+CD25+Foxp3+ T regulatory cells as well as Th2 cytokines without affecting IL-9 in the lung. These results suggest a role of Tyk-2 in different subsets of T helper cells mediated by SOCS3 regulation that is relevant for the treatment of asthma, cancer and autoimmune diseases.

In mammals cytokines signal primarily through signaling pathways involving cytokine-receptor-activated Janus kinases (JAKs 1–3 and Tyrosine kinase 2) and STAT (Signal and Activators of Transcription 1–6) transcription factors. Tyk-2 has been first described by Velazquez et al. and it is known to be involved in interleukins IL-6, IL-10, IL-11, IL-12, IL-22, IL-23, IL-27, IL-31 and IFN (interferon) α, β and γ signal transduction. However, IL-6 and IL-10 mediated signal transduction is still functional in Tyk-2(-/-) mice. Tyk-2 deficiency in humans has been found to be associated with an increased susceptibility to viral and mycobacterial infections as well as atopic dermatitis, hypereosinophilia and Hyper IgE syndrome. These symptoms hint towards a Th2-skewed cytokine profile in the absence of Tyk-2 function. As these cytokines play a major role in the pathogenesis of allergic diseases such as asthma, we analyzed Tyk-2(-/-) mice in a murine model of allergic asthma.

Allergic asthma is an inflammatory disease of the airways which occurs with increased prevalence during the last decades especially in the developed world. However, the molecular mechanism of this disease needs further investigations. Recently, the role of IL-17A and IL-9 in an experimental model of asthma has been further explored. IL-9 was first characterized as a T cell and mast cell growth factor. Transgenic expression of IL-9 in the lung results in allergic inflammation dependent on Th2 cytokines. Moreover, blockade of IL-9 inhibits mastocytosis and airway remodeling. Th9 cells are induced by TGF-β and IL-4.

IL-17A was found to play a pathogenetic role during the developmental phase of the disease, while it ameliorates established allergic disease. Since IL-17A is a known neutrophil attracting cytokine, it has been hypothesized that it is more associated with neutrophil-dominated allergic asthma. This form of the disease is known to be more severe and difficult to treat than eosinophil-dominated asthma. Additionally, IL-17A has also been identified to be a main causative agent for the occurrence of airway hyperreactivity, one of the key hallmarks of allergic asthma. The suppressor of cytokine signalling (SOCS)-3 activation, inhibits several cytokine signaling in T helper cells as well as in dendritic cells thus regulating and having a significant impact on the development of the downstream immune responses.

Here, we show that Tyk-2(-/-) mice have hypereosinophilia and hyper IgE associated with increased remodeling, mast cells, effector Th2 and Th9 cells in the airways. This phenotype was found to be associated with a defect in Th17 cell polarization. Moreover, in the lung of asthmatic Tyk-2 deficient mice, protein level was found upregulated. Treatment with rIL-17A led to a down-regulation of Th2 and T regulatory cells in the lung of Tyk-2(-/-) mice. In conclusion, Tyk-2 plays a fundamental role downstream of many cytokine receptors in controlling allergic asthma by limiting different subsets of T helper cell derived cytokines such as IL-6, IL-9 and IL-21 and...
inducing IL-17A and IL-17F. Tyk-2 in asthma probably regulates these cytokines in the presence of suppressed levels of SOCS3.

Results

Tyk2 deficiency leads to a severe Th2 allergic phenotype after allergen challenge. Allergic asthma induced in Tyk2 deficient mice, leads to severe infiltration of eosinophils in the BALF as compared to the wild type littermates mice (Fig. S1a–c). This effect is probably mediated by the increase of IL-5, the eosinophil growth and differentiating factor (Fig. S1 d). Tyk2 deficiency is also known to be associated with hyper IgE production7 (Fig. S1e). Along with hyper-IgE these mice also displayed elevated levels of IgG2A compared to wild type mice (Fig. S1f). In spite of elevated Th2 cytokines and inflammation in the airways, Tyk-2 deficient mice did not show increased airway hyperresponsiveness to methacholine after allergen sensitization and challenge (Fig. S1g).

We also detected induced fibrosis in the lung of Tyk2(-/-) mice after allergen sensitization and challenge (Fig. S1h). Consistent with these findings, lung CD4+ T cells isolated from the Tyk-2 deficient mice released increased amounts of IL-13, a Th2 cytokine which favors fibrosis, as compared to wild type littermates (Fig. S1i).

Increased Th9 and mast cell number in the lung of Tyk-2(-/-) mice. Mast cells are major players in the induction of allergic diseases such as asthma. Since it has been previously described that Tyk-2(-/-) mice show an increased Th2 differentiation and thus a more severe asthmatic phenotype21, we analyzed the number of mast cells in Tyk-2(-/-) mice after allergen challenge. In a murine model of allergic asthma (Fig. 1a), we thus analyzed the lung section stained with Toluidine Blue, a specific staining for mast cells, and found an increased number of positive cells in the absence of Tyk-2 (Fig. 1b). We then analyzed total lung cells by flow cytometry. Mast cells were defined as c-kithiCD123^*FcεRI^* cells gated on SSC^hi^c-kit^hi^. These mast cells are known as mucosal mast cells as they express the IL-3R (CD123). The analysis showed a significant up-regulation of mast cells in Tyk-2(-/-) mice after allergen challenge (Fig. 1c) identifying mucosal mast cell number as a possible explanation for the allergic phenotype of these mice.

Finally, we looked at the concentrations of the two main mast cell-inducing cytokines, IL-3 and IL-9. Further analysis showed that lung CD4+ T cells from Tyk-2(-/-) mice released significantly more IL-3 (Fig. 1d) and IL-9 (Fig. 1e) as well as IL-10 (Fig. 1f and 1g) after ovalbumin challenge. Analysis of the recently found Th9-defining transcription factor interferon-regulatory factor 4 (Irf4), which also
regulates Th2 and Th17 cell differentiation also revealed induced expression levels after allergen challenge in Tyk-2(-/-) mice compared to wild-type mice in total lung (Fig. 1h). Finally, the most recognized Th9 transcription factor Pu.1 was also found up-regulated at the mRNA level in the lung of Tyk-2 deficient mice (Fig. 1i). These data show an increased number of mast cells and mast cell growth factors such as IL-3 and IL-9 in the lung of Tyk-2(-/-) mice.

Increased proliferation of lung CD4+ T cells isolated from Tyk-2(-/-) asthmatic mice. To investigate the role of Tyk-2 in T cells in a setting of allergic asthma, we sorted out lung CD4+ T cells from asthmatic mice and analyzed their proliferation after CFSE staining in a four days cell culture. As shown (Fig. 2 a and b), CD4+ T cells isolated from the lung of Tyk-2(-/-) mice proliferated more than those from the lung of wild type littermates, indicating increased effector T cells in the absence of Tyk-2. We then analyzed IL-6, a T regulatory cell inhibiting cytokine, and found it elevated in the supernatants of cultured lung CD4+ T cells in the absence of Tyk-2 in experimental allergic asthma (Fig. 2c). Similarly, IL-21, a cytokine known to counteract T regulatory cells, was found increased in the supernatants of lung CD4+ T cells of Tyk-2(-/-) mice (Fig. 2d), while TGF-β, a T regulatory inducing cytokine was found unchanged before and after allergen challenge (Fig. 2e,f). The Th9 subset differentiates in vitro by the addition of TGF-beta and IL-4. We next analyzed IL-4 which, consistent with a role of Tyk-2 in Th2 cells, was found significantly up-regulated in the supernatants of lung CD4+ cells isolated from the Tyk-2(-/-) mice as compared to those isolated from wild type littermates (Fig. 2g). Taken together, these data support a central role of Tyk-2 in inhibiting Th9 differentiation and in general its T cell anti-proliferative function in allergic asthma.

Increased SOCS3 in the lung of Tyk-2(-/-) mice in a murine model of allergic asthma. IL-9 was originally identified as a T cell growth factor and it is a member of the common-c-chain-receptor cytokine family including IL-2, IL-4, IL-7, IL-15 and IL-21. The activation of IL-9 receptor α-γ-chain induces activation of JAK1-3 and downstream STAT1/3 and STAT5. It has been demonstrated that IL-9 induces SOCS3 which then limits IL-9 signaling. Considering that IL-6, IL-21, IL-4 and IL-9 were found elevated in asthma
particularly in the absence of Tyk-2, we then looked at the downstream signal of these cytokines. First, we looked at pSTAT3 which is activated by IL-9 and IL-6. Although it was found elevated in the lung of Tyk-2(−/−) asthmatic mice, no difference was observed between wild type and Tyk-2 deficient mice (Fig. 2h).

STAT5 mRNA was also not found significantly regulated by Tyk-2 in this disease model (Fig. 2i). By contrast, SOCS3 protein was found significantly up-regulated in the lung of asthmatic Tyk-2(−/−) mice (Fig. 2j).

Altogether, these data underline a role of IL-9 mediated by SOCS3 in the lung of Tyk-2 deficient mice.

Tyk-2 deficiency is associated with reduced IL-17A and IL-17F production after allergen challenge. We next analyzed IL-17A and IL-17F levels which we found to be down-regulated in Tyk-2(−/−) mice after allergen challenge (Fig. 3a, b) as well as in naive mice (Fig. 3c) compared to wild-type mice.

Next, we looked at the production of the cytokines relevant for Th17 development. IL-1β production was analyzed in total lung cells. We could observe a significant increase of this cytokine after allergen challenge in Tyk-2(−/−) mice (Fig. 3d). Furthermore, in the BALF of naive Tyk-2(−/−) mice we could detect higher amounts of IL-21 (Fig. 3e) and IL-23 (Fig. 3f) than in naive wild-type mice. After allergen challenge, IL-21 and IL-23 were found down-regulated especially in the BALF of Tyk-2(−/−) asthmatic mice. These data on IL-21 are similar to the findings on IL-10. In fact, IL-21 was found up-regulated in the supernatants of lung CD4+ T cells after allergen challenge but down-regulated in the BALF. This might be due to IL-21 release by different cell types.

Th17 differentiation is impaired in Tyk-2(−/−) mice. Tyk-2 is located downstream of IL-6 and IL-23 signaling that are crucially involved in the generation and proliferation of Th17 cells30,31. We thus wanted to determine whether alternative pathways were involved in the development of Th17 cells in the absence of Tyk-2. To this aim we isolated splenic CD4+CD62L− T cells and exposed them to Th17 skewing cell culture conditions (α-CD3, α-CD28,

Figure 3 | Tyk-2(−/−) mice showed a defective Th17 cytokine production in vivo. (a). and (b). Mice were treated with OVA as described in the methods section. After 21 days the mice were sacrificed and total cells were isolated from the lung. After 24 h incubation with antibodies against CD3 and CD28 supernatants were analyzed by ELISA for IL-17A ((a): n = 2–4, p = 0.041; p = 0.007) and IL-17F ((b): n = 2–4, p = 0.046, p = 0.046). (c). Total lung cells from untreated mice were isolated and incubated with antibodies against CD4 and IL-17A and analyzed by flow cytometry. Data show on the one hand lung IL-17A+CD4+ T cells (left panel n = 3–4, p = 0.003) and on the other hand lung IL-17A+CD4+ cells (right panel n = 3–4, p = 0.003). (d). Total lung cells from naive and allergen-treated mice were incubated with α-CD3 and α-CD28 antibodies for 24 h and analyzed by ELISA for IL-1β production (n = 2–4, p = 0.009), (e). and (f). IL-21 (n = 4–5, p = 0.025; p = 0.0019; p = 0.041; p = 0.002) and IL-23 (n = 3–5, p = 0.045; p = 0.038; p = 0.043; p = 0.012) levels were measured in the BALF of naive and asthmatic mice.
Our data demonstrates that the exogenous IL-17A can induce neutrophilia in the lung in the presence or absence of Tyk-2 and inhibit T regulatory cells downstream of the IL-17A receptor. When we analyzed the samples for IL-9 expression, we could detect similar IL-9 levels in lung CD4 T cells (Fig. 5g) irrespective of the treatment with rIL-17A. Taken together, these data demonstrate that the exogenous IL-17A can induce neutrophilia in the lung in the presence or absence of Tyk-2 and inhibit T regulatory cells in the absence of Tyk-2.

**Discussion**

Here, we report a crucial protective regulatory role of Tyk-2 in allergic asthma. In this study, we analyzed the role of Tyk-2 on IL-9 levels in lung CD4 T cells (Fig. 5g) irrespective of the treatment with rIL-17A. Taken together, these data demonstrate that the exogenous IL-17A can induce neutrophilia in the lung in the presence or absence of Tyk-2 and inhibit T regulatory cells in the absence of Tyk-2.

Figure 4 | Tyk-2(-/-) naive CD4+ T cells showed a defective Th17 cytokine production ex vivo. Spleen CD4+CD62L+ T cells were isolated from naive Tyk-2(-/-) and wild-type mice and treated with antibodies against CD3 (2 μg/ml), CD28 (2 μg/ml), IL-4 (10 μg/ml) and INFγ (10 μg/ml) while recombinant cytokines TGF-β (3 ng/ml), IL-6 (20 ng/ml), IL-23 (50 ng/ml) and IL-21 (80 ng/ml) were added as indicated. After five days the supernatant was collected and analyzed by ELISA for IL-17A ((a): n = 2, p = 0.004, p = 0.009, p = 0.0008, p = 0.0003, p = 0.006) IL-10 ((b): n = 2, p = 0.003) and IL-17F ((c): n = 2, p = 0.001, p = 0.004, p = 0.0003).

Treatment of Tyk-2(-/-) mice with recombinant IL-17A in a murine model of asthma reduced T regulatory cells. To analyze the role of IL-17A deficiency in the absence of Tyk-2, we thought to treat Tyk-2 deficient mice intranasally with recombinant murine IL-17A in a murine model of allergic asthma.

Intranasal application of rIL-17A (Fig. 5a) led to a down regulation of the Th2 cytokines IL-4, IL-5 and IL-13 only in Tyk-2(-/-) mice (Fig. 5b–d, respectively). IL-17A is important for the recruitment of neutrophils to the lung during allergic airway disease. Here, we describe for the first time the number of these cells remained unchanged in wild-type mice (Fig. 5f). Thus, Tyk-2(-/-) mice had fewer regulatory T cells after IL-17A treatment than wild type mice consistent with a role of Tyk-2 in the generation of T regulatory cells downstream of the IL-17A receptor.

When we analyzed the samples for IL-9 expression, we could detect similar IL-9 levels in lung CD4+ T cells (Fig. 5g) irrespective of the treatment with rIL-17A. Taken together, these data demonstrate that the exogenous IL-17A can induce neutrophilia in the lung in the presence or absence of Tyk-2 and inhibit T regulatory cells in the absence of Tyk-2.

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elevation of SOCS3 in the lung of Tyk-2 deficient mice. SOCS3 is thought to play an important role in determining T cell responses34. The data presented in this manuscript are consistent with an elevation of SOCS3 in DCs which limits the tolerogenic effect of DCs thus inducing T effector cells20,35.

SOCS3 deficient DCs have been shown to give rise to more effective T regulatory cells. Thus SOCS3 induces the development of more effector T cells such as Th2 and Th9 in the absence of Tyk-2. By contrast, the T regulatory cells are inhibited in the presence of SOCS3 in DCs.

IL-10 was induced in lung CD4 T cell supernatants from Tyk-2(+/−) mice after allergen challenge. IL-10 is a cytokine which is also produced by T regulatory cells, Th2, Th9 and Th17 cells.

Mast cells are an important cell type in the pathophysiology of allergic asthma. We then analyzed the presence of mast cell inducing cytokines IL-3 and IL-9 and found that they were elevated in Tyk-2(+/−) mice compared to wild type mice after allergen treatment. Consistently, we could also detect an increase in mast cells in the lungs of these mice. IL-9 has been described to induce SOCS3 which in turn inhibits IL-9. However, considering that mast cells as well as T effector cells develop in the absence of Tyk-2, it is possible that SOCS3 cannot inhibit IL-9 in Tyk-2(+/−) mice. In fact, SOCS3 binds to Tyk-2 and JAK2/JAK1 downstream of IL-9 and other cytokines using the same receptor γ chain8,20,28. It could be already demonstrated that IL-21 is expressed in activated human CD4+ T cells but not in most other tissues. Furthermore, the expression of IL-21 is up-regulated in Th2 and Th17 subsets of T helper cells, as well as T follicular helper (Tfh) cells. In addition, IL-21 is expressed in NKT cells regulating the function of these cells. A study using mice with peanut allergies showed that systemic treatment with IL-21 effectively weaked the allergic response36–39. In conclusion, the low levels of IL-21 in the BALF in asthma could be consistent with the decreased production of this cytokine by NKT cells thus favoring IgE development in allergy. By contrast, the increase of IL-21 in lung CD4 T cell supernatants might reflect the expansion of the Thf and Th2 cells in asthma. These Th17 cells however cannot be supported by IL-23 because it was found decreased especially in Tyk-2(+/−) asthmatic mice.

IL-17A has been described to play a role in inducing airway hyperresponsiveness40. Since Tyk-2(+/−) mice show a much more severe asthmatic phenotype but no elevated AHR compared to wild-type mice we applied this cytokine during allergen challenge. Wild-type mice showed increased neutrophils in the BALF compared to Tyk-2(+/−) mice after allergen challenge. Treatment with recombinant IL-17A led to an increase in BAL neutrophils in both mouse strains compared to respective OVA treated mice. However, this effect was significantly stronger in wild type mice suggesting a reduced recruiting capacity in Tyk-2(+/−) mice which was not rescued by

**Figure 5** | Treatment with rIL-17A led to down-regulation of T regulatory cells in Tyk-2 deficient mice. (a). Mice were treated with OVA alone and OVA + rIL-17A as shown in the experimental design. (b).–(d). Lung CD4+ T cells from OVA alone and OVA + rIL-17A treated mice were isolated and incubated for 24 h with α-CD3 and α-CD28. The supernatants were analyzed by ELISA for IL-4, IL-5 and IL-13 ((b):IL-4: n = 3–4; p = 0.004; p = 0.003; (c):IL-5: n = 3–4; p = 0.002; p = 0.040; (d): IL-13: n = 3–4; p = 0.002; p = 0.018; p = 0.017). (e). BALF was retrieved and BAL cells were incubated with antibodies against CD3, CD45R, CCR3 and Gr-1 and subsequently analyzed by flow cytometry. CD3+CD45R+ CCR3+Gr-1+ cells were counted as neutrophils. (f). Total lung cells from OVA and OVA + rIL-17A treated mice were incubated with antibodies against CD4, CD25 and Foxp3 and analyzed by flow cytometry (n = 3–4; p = 0.001; p = 0.002). (g). Lung CD4+ T cells from OVA alone and OVA + rIL-17A treated mice were isolated and incubated for 24 h with α-CD3 and α-CD28. The supernatants were analyzed by ELISA for IL-9 (n = 3–4; p = 0.0904; p = 0.016).
exogenous IL-17A. Moreover, IL-17A treatment led to a reduction in Th2 cytokine production in Tyk-2−/− mice while the production was not altered in wild type mice. In accordance with the findings from Betelli and co-workers we think that IL-17A is able to block Th2 cytokine production in a Tyk-2-dependent manner. Also, IL-17A led to a down-regulation of regulatory T cells in Tyk-2−/− mice, while their number in the wild-type littermates remained unchanged. These data are consistent with an up-regulation of SOCS3 in the lung of Th2 deficient mice. IL-9 was found unchanged in lung CD4+ T cells before and after in vivo RII-17A treatment of Tyk-2−/− mice suggesting that IL-17A does not influence directly IL-9 production in T cells.

Altogether, these results demonstrate a reduced survival fitness of the Th17 in the absence of Tyk-2 mediated by the up-regulation of SOCS3 that should be considered for a therapeutic setting in allergy as well as in autoimmune diseases and cancer.

Methods

All methods were carried out in accordance with the approved guidelines. Mice. Tyk-2−/− and wild-type mice on a Balb/c genetic background were used at the age of 6 to 8 weeks. All experiments were undertaken with the approved licenses (23-177-07/G09-1-008, 23-177-07/051-1V2 from ethical Review Board; Rheinland-Pfalz). The age of 6 to 8 weeks. All experiments were undertaken with the approved licenses (23-177-07/G09-1-008, 23-177-07/051-1V2 from ethical Review Board; Rheinland-Pfalz). The age of 6 to 8 weeks. All experiments were undertaken with the approved licenses (23-177-07/G09-1-008, 23-177-07/051-1V2 from ethical Review Board; Rheinland-Pfalz). The age of 6 to 8 weeks. All experiments were undertaken with the approved licenses (23-177-07/G09-1-008, 23-177-07/051-1V2 from ethical Review Board; Rheinland-Pfalz).

OVA sensitization and challenge. Female Tyk-2−/− and wild-type mice on a Balb/c background were maintained specific pathogen-free conditions and received i.p. injections of 500 µg/ml ovalbumin (Calbiochem, Gifhorn) complexed with 10% alum (Sigma-Aldrich, Steinheim, Germany) on days 0 and 7 and previously described. On days 18, 19 and 20 the animals received ovalbumin in PBS via aerosol (10 mg ovalbumin/ml PBS in aerosol solution) for 30 minutes in a chamber connected to an aerosol generator (Föhr Medical Instruments, Seeheim, Germany). Twenty-four hours after the last aerosol, invasive whole-body plethysmography was performed to measure airway resistance and mice were sacrificed afterwards to isolate lung or spleen cells as previously described. For recombinant IL-17A treatment mice were anaesthetized 60 minutes before allergic challenge was performed and given 100 ng (IL-17A) intranasally on days 18, 19 and 20.

Assessment of AHR and collection and analysis of bronchoalveolar lavage. To measure airway reactivity, we used whole-body plethysmography as previously described. Data are expressed as mean values of “airway resistance [R]/baseline RT” ± SEM. After plethysmography, bronchoalveolar lavage of the right lung was performed twice with 800µl saline each. BALF supernatants were frozen and subsequently analyzed by ELISA. The cell pellet was resuspended in PBS and counted (CASY® TT, Roche Diagnostics, Mannheim, Germany) and subsequently used for cytospin analysis after staining with May-Grünwald-Giemsa solution (Carl Roth, Karlsruhe, Germany) for Flow Cytometric Analysis (FACS).

Histology. Lungs were removed, fixed in 10% formalin-PBS solution, dehydrated and embedded in paraffin. 5 µm thick sections were stained with Haematoxylin and Eosin Gomori’s solution (Carl Roth, Karlsruhe, Germany) or for Flow Cytometric Analysis (FACS).

Isolation and analysis of lung and spleen CD4+ T cells and cell culture. Lung CD4+ T cells were positively selected by magnetic bead isolation (MACS, Miltenyi Biotec, Bergisch-Gladbach) according to the manufacturer’s protocol. In some experiments lung and spleen CD4+ T cells were incubated with anti-CD3 (2 µg/ml), anti-CD28 (2 µg/ml). Antibodies were from hydridoma cell cultures. After 24 hours, or later as indicated, the supernatants were removed and analyzed by ELISA and RNA extracted from the cells.

Th17 skewing of spleen cells. Spleens were removed from naïve Tyk-2 and Balb/c mice and CD4+ CD62L− were positively selected by magnetic bead isolation (MACS, Miltenyi Biotec, Bergisch-Gladbach) according to the manufacturer’s protocol. Cells were plated at a concentration of 1 Million per ml and were incubated with plate-bound anti-CD3 (2 µg/ml), anti-IL-4 (10 µg/ml), anti-IFNγ (10 µg/ml), TGF-β (3 ng/ml) and anti-CD28 (2 µg/ml) antibodies. Additionally cells received 20 ng/ml recombinant IL-6 with or without 80 ng/ml of recombinant IL-2 or 50 ng/ml of recombinant IL-23. After three days IL-2 was added and day 5 supernatants were frozen.

Flow cytometric analysis, intracellular staining and proliferation assay. Total lung cells were stained with antibodies for 30 min at 4°C, washed in PBS, and then analyzed on a FACS-Calibur (BD Pharmingen, Heidelberg, Germany). The following FITC-, PE-, PerCP or APC-conjugated monoclonal antibodies were used for flow cytometry: CCR3, CD3, CD4 (L3T4), CD25, CD45R, Gr-1, Foxp3, IL-17A, ckit and FcεRI, Antibodies were purchased from BD Pharmingen (BD Pharmingen, Heidelberg, Germany), Miltenyi Biotec (Miltenyi Biotec, Bergisch-Gladbach) or ebioscience (ebioscience, Frankfurt, Germany).

For intracellular staining for Foxp3 cells were stained with anti-CD4 (L3T4; BD Pharmingen) and anti-CD25 (BD Pharmingen, Heidelberg, Germany) antibodies for 30 min at 4°C. Afterwards, cells were fixed with fixation/permeabilization solution (ebioscience, Frankfurt, Germany) for 35 min at 4°C and washed with permeabilization buffer (ebioscience, Frankfurt, Germany). Cells were then incubated with antibodies against Foxp3 (Miltenyi Biotec, Bergisch-Gladbach, Germany) for 30 min at 4°C in permeabilization buffer and washed once.

For staining of IL-17A, freshly isolated lung cells were incubated over night with anti-CD3 and anti-CD28 at 37°C. Then they were stimulated with 1 ng/ml recombinant IL-23 (Sigma-Aldrich, Steinheim, Germany), and GolgiStop (BD Pharmingen, Heidelberg, Germany) for 4 h. After harvesting cells, the same protocol as for Foxp3 staining was used.

Furthermore, a proliferation assay with CD4+ lung cells was performed as previously described.

ELISA. Mouse IL-3 (7.8–500 pg/ml), IL-4 (7.48–500 pg/ml), IL-5 (1.56–1000 pg/ml), IL-6 (15.6–1000 pg/ml), IFNγ (1.0–100 ng/ml), IL-12 (3.1–200 ng/ml) and IL-10 (31.3–2000 pg/ml), were detected by using a specific sandwich ELISA (OptEIA; BD Pharmingen, Heidelberg, Germany). ELISA kits for mouse IL-1β (15.6–1000 pg/ml), IL-12 (15.6–1000 pg/ml), IL-17A (15.6–1000 pg/ml) and IL-17F (23.4–1500 pg/ml) were from R&D Systems, Wiesbaden, Germany. IL-9 (30–400 pg/ml) was detected using a Readyto-use kit from ebioscience (Frankfurt, Germany).

qPCR. Lungs were removed and stored in preqGold RNA Pure (peqlab, Erlangen, Germany) at −80°C until analysis. Tissue was homogenized in preqGold RNA Pure and used for RNA extraction according to the manufacturer’s protocol. Cells were also lysed directly on the cell culture plate and taken for RNA extraction. Briefly, cells were treated with chloroform (Roth, Karlsruhe, Germany) and RNA was precipitated with isopropanol (Roth, Karlsruhe, Germany). RNA concentration in each sample was determined using a spectrophotometer (Nanodrop, peqlab, Erlangen, Germany). Twenty ng of cDNA was used in each reaction and added to 2× SoFast™ EvaGreen® Supermix (BIO-RAD, Munich, Germany) and 200 nM primer. Murine primers for IL-9 (fwd: 5′-CGT ATG ATT GTA CCA CGC CGT GC-3′, rev: 5′-GCC TTT GCA TCT TGT TCT GG-3′), IFNγ (fwd: 5′-AGG CCT CGC TCT AGG TCG TCA AAC-3′, rev: 5′-AGC ATC TTC TCG ACC CTG AA-3′), Tbet (fwd: 5′-CCA AGA CCC TCC TAA AGA AC-3′, rev: 5′-AGC TTG TCA TGG ACC CTG TG-3′), Pdl1 (fwd: 5′-GCA TGG GTG TGG TGG ACA A, rev: 5′-TCT TGG CAG TGT TGC CCA G), Sox3 (fwd: GTT CCT GGA TCA TGA TGA TGC, rev: GCG TGT AGA TGA GTA TGC TGC, rev: GCAC TGT GCC TTA CCT CAC TGA) and as control Hprt (for: 5′-GCC CCA AAA TGA TTA TGA AGG TT-3′, rev: 5′-TTG GGC TTA TCT TAC GTT CT-3′) were purchased from Eurofin-MWG-Operon, Ebersberg, Germany. qPCR was run on CFX96 Real-Time PCR Detection System (BIO-RAD, Munich, Germany), and analyzed with CFX Manager Software.

Statistical analysis. Differences were evaluated for significance (* p < 0.05; ** p < 0.01; *** p < 0.001) with the Student-two-tailed t test for independent events by using statistical analysis (Excel, Microsoft, version 2003). Data are given as mean values ± SEM.
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Acknowledgments
We thank D. Engelbrecht and A. Geiger for animal husbandry, and S. Trump, M. Mathews, M. Schubert, A. Mättl for their technical assistance. We thank S. Mousset for graphic improvement of the figures. This work was supported by the department of Molecular Pneumology in Erlangen. Initially C.U.’s PhD was supported by a DFG and the Graduiertenkolleg GRK 1043 “Antigen-specific Immunotherapy” in Mainz and later by a DFG grant in Erlangen (Germany). Mice were originally provided to us by Mathias Müller (University of Veterinary Medicine, Vienna). A.G. is supported by the EU grant Predicta. We acknowledge support by Deutsche Forschungsgemeinschaft and Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU) within the funding programme Open Access Publishing.

Author contributions
C.U. and S.F. designed the experiments; C.U. and A.G. performed the experiments; R.J.R. and H.-A.L. performed histological analysis; C.U., A.G. and S.F. wrote the paper; and M.M., A.G. and S.F. edited the paper.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Ubel, C. et al. Role of Tyk-2 in Th9 and Th17 cells in allergic asthma. *Sci. Rep.* **4**, 5865; DOI:10.1038/srep05865 (2014).

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