Increased expression of the Th17-IL-6R/pSTAT3/BATF/RorγT-axis in the tumoural region of adenocarcinoma as compared to squamous cell carcinoma of the lung

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Here we describe increased expression of IL6R in the tumoural region of lung tissue from patients affected by lung adenocarcinoma as compared to squamous cell lung carcinoma. Moreover, here we found increased IL6R in the tumour free part of the lung. By using a murine model of lung adenocarcinoma, we discovered that few lung tumour cells expressed IL-6R and CD4+CD25+Foxp-3+ T regulatory cells down-regulated IL-6R in the tumour bearing lungs. Downstream of IL-6R, the Th17 lineage-specification factors: Signal transducer and activator of transcription 3 (STAT3), Basic leucine zipper transcription factor, BATF and a protein encoded by the RORC in human (RAR-related orphan receptor C) (RORcT), were also found induced in the tumoural region of lung tissue from patients affected by lung adenocarcinoma as compared to those carrying squamous cell carcinoma. Moreover, pSTAT3 protein was found phosphorylated and auto-phosphorylated in the tumoural region of patients with adeno cell carcinoma of the lung as compared to the tumoural region of patients with squamous cell carcinoma of the lung. Intranasal application of anti-IL-6R antibodies in a murine model of lung adenocarcinoma, induced T regulatory cell markers such as Foxp3, Ctl4, Icos, Il10, Il21, Folr4 and Lag3 and inhibited Rorc in lung adenocarcinoma.

lung cancer is the leading cause of cancer deaths world-wide and lung adenocarcinoma is one of the predominant histological subtypes of lung cancer (40%) followed by squamous (30%) and large cell lung carcinoma among non-small-cell lung cancers (NSCLC), which represent 85% of all lung tumours. The development of these tumours is not only initiated by smoking but can be also attributed to genetic spontaneous mutations. Epithelial cells are initially transformed into lung carcinomas. Most of lung cancer cell lines including those with Epidermal-growth factor receptor (EGFR) mutations harbour auto-phosphorylated STAT3 that cannot be inhibited by EGFR inhibitors. By contrast, inhibitors of JAK1 and IL-6 resulted in reduced lung tumour cell growth and inhibition of STAT3 phosphorylation. We have previously demonstrated that delivery of anti-IL-17A antibody could successfully reduce lung tumour load, resulting in up-regulation of the Th1/Tc1 cytotoxicity and reduction of immunosuppressive regulatory CD4+CD25+Foxp3+ T cells in a murine model of lung adenocarcinoma. These data indicate Th17 cells as a potential target for immunotherapy against lung adenocarcinoma.

IL-6 along with TGF-β, which is present in the tumour microenvironment, induces Th17 cells and IL-6+ mice have reduced Th17 cell development. After binding of IL-6 to its receptor (IL-6R), JAK1-Tyk-2 and STAT3 are activated, resulting in STAT3 phosphorylation-dimerization and translocation into the nucleus. Consistent with a role of STAT3 in Th17 differentiation, STAT3-Cd4- conditional deficient mice have reduced Th17 cells. Finally, downstream of IL-6, STAT3, RORγT and BATF cooperate to induce IL-17A gene.
BATF is a newly described transcription factor crucial for the differentiation of IL-17A-secreting Th17 cells, which play a pathogenic role in lung adenocarcinoma development. BATF is a new member of the ATF/CREB family of transcription factors that has been isolated from a cDNA library prepared from Epstein-Barr virus stimulated human B cells and that showed the strongest hybridization in lung and Raji Burkitt’s lymphoma by Northern blot analysis. More than ninety percent of mice overexpressing human BATF in T cells by 1 year of age develop a lymphoproliferative disorder (LPD). Moreover, the human oncoprotein JunB is a specific binding partner of BATF.

We thus thought to clarify the role of IL-6 signaling on the Th17 pathway in lung cancer tumour-infiltrating lymphocytes (TIL) as well as in lung cancer cells in different subtypes of NSCLC, considering that STAT3 is also an oncogene whose activation has been linked to EGFR mutation, which are present in lung cancer.

**Results**

**Analysis of the cohorts of patients with adenocarcinoma versus squamous carcinoma of the lung.** The clinical characteristics of the patients analysed in this study are reported in Table I.

In this study, we isolated mRNA and proteins from the tumoural and control regions (tumour-free) of two cohorts of patients with squamous cell carcinoma (SCC) and adenocarcinoma (ADC) of the lung. Moreover, we performed cryosections of the tumoural and control lung regions from OCT frozen lung tissues. The control region was taken at least 5 cm far away from the outer edge of the tumoural region (Fig. S1).

IL-6R is increased in the control region of NSCLC and in the tumoural region of ADC as compared to the tumoural region of SCC. IL-6 signals after its binding to the IL-6 receptor (IL-6R) alpha chain. We thus analysed the expression of the IL-6RA mRNA in NSCLC. Here we found a significant up-regulation of IL-6RA mRNA in the control region of both adeno- and squamous cell carcinoma as compared to the respective tumoural region (Fig. 1a). Moreover, IL-6RA mRNA was found significantly induced in the tumoural region of ADC as compared to the tumoural region of SCC (Fig. 1a, right handside). Altogether, these data indicate that IL-6RA mRNA expression is induced in the tumour region of adenocarcinoma bearing lungs as compared to the tumoural region of SCC. Moreover, there seems to be in both histological types of cancer a down-regulation of IL-6R expressing cells in the tumoural region. This could also be confirmed by immunohistochemical staining of IL-6R on tissue sections from the control and the tumoural lung region of patients with squamous as well as adenocarcinoma (Fig. 1 c).

The tumour cells used to induce experimental lung adenocarcinoma do not express IL-6R. To start to investigate whether the down-regulation of IL-6R in the tumoural region was due to the lack of IL-6R in the tumour cells, we analysed IL-6R expression by flow cytometry in the murine tumour cell line LL2/2-luc-M38, used to induce experimental lung adenocarcinoma (Fig 1b). As shown, the lung adenocarcinoma cell line did not express IL-6R as this staining was comparable to the negative control. These findings probably explain why IL-6RA is down-regulated in the tumoural region of the patients with NSCLC.

CD4+CD25+FOXP-3+ T regulatory cells in lung adenocarcinoma tumour bearing mice down-regulated IL-6R. We have previously demonstrated that IL-6R alpha chain is expressed on lung CD4+CD25+Foxp3+ T regulatory cells and that blockade of IL-6R induces T regulatory cells. Lung adenocarcinoma is characterized by increased FOXP-3, which is a transcription factor signature of T regulatory cells. We thus looked in the murine model of lung adenocarcinoma at the distribution of IL-6R expression in CD4+ T effector and CD4+CD25+FoxP-3+ T regulatory cells. Here we found that the number of lung CD4+ T cells bearing the IL-6R did not change in the lungs of naive mice as compared to those present in the lungs of mice bearing tumour (Fig. 2a). However, we found a significant down-regulation of IL-6R alpha expression on the CD4+CD25+FOX-P3+ T regulatory cells present in mice bearing tumour (Fig. 2b).

In conclusion, in mice bearing tumour both tumour cells and CD4+CD25+FOX-P3+ T regulatory cells down-regulate IL-6R.

Increased BATF gene expression in the tumoral region of patients with lung adenocarcinoma (ADC) as compared to squamous cell carcinoma (SCC). Downstream of IL-6R, BATF is activated. We thus asked if BATF was also up-regulated in the adenocarcinoma. Here we found that BATF mRNA is significantly increased in the tumour region of patients affected by lung adenocarcinoma as compared to the level measured in tissue derived from the tumour region of patients affected by lung squamous cell carcinoma (Fig. 3a, right hand side), suggesting a local induction of Th17 responses specific for adenocarcinoma but not for squamous cell carcinoma in the tumoural region. Consistently, by immunohistochemistry, BATF protein was found significantly up-regulated in the tumour region of patients with adenocarcinoma as compared to the tumoural region of patients with squamous cell carcinoma (Fig. 3b and 3c, respectively). Interestingly, in the control region of the lung of patients with squamous cell carcinoma, BATF was significantly induced as compared to the tumoural region (Fig. 3a). No difference in the distribution of BATF mRNA was observed in adenocarcinoma patients between control and tumoural regions.

Finally, in a murine model of lung adenocarcinoma, we found that CD4+ T cells, isolated from the lungs of mice bearing lung cancer cells, expressed significantly elevated levels of Batf mRNA as compared to those isolated from the lungs of naive mice (Fig. 3 d–e).

Increased phospho- and autophosphorylation of STAT3 in the tumoral region of lung tissue from patients affected by adenocarcinoma as compared to those with squamous cell carcinoma. Downstream of IL-6R, STAT3 is activated. Subsequently, the gp130 tails are phosphorylated, which mediates the recruitment of the STAT3 proteins. Dimerization of activated (phosphorylated) STAT3 is followed by nuclear entry. Within the nucleus, STAT3 can enhance the transcription of many genes, including those that encode acute-phase proteins. Consistent with the up-regulation of Batf, we found an up-regulation of Stat3 mRNA in the lungs of tumour bearing mice as compared to tumour free animals (Fig. 3f).

Similarly, we next found an up-regulation of Stat3 mRNA in the tumoral region of lung adenocarcinoma as compared to the tumoral region of squamous carcinoma (Fig. 4a). Moreover, we found a down-regulation of Stat3 mRNA in the tumoral region of squamous cell carcinomas compared to the respective control region. By contrast, Stat3 mRNA was comparable in the control and tumoral region of the adenocarcinoma groups (Fig. 4a). We next thought to investigate if there was an auto activation of STAT3 ongoing in the tumoral region of adenocarcinoma. To do so, we analysed proteins isolated from the different regions of NSCLC by western blot analysis. We then analysed the phosphorylation state of STAT3 in these tissues and found significantly increased phosphorylation of STAT3 at both tyrosine (pY-STAT3: phospho-tyrosine) (Fig. 4b and f) and serine (pS-STAT3: phosphoserine) (Fig. 4c and e) residues, in the tumoral region of adenocarcinoma as compared to squamous carcinoma (Fig. 4f and Supplementary Fig. 2).

In conclusion, STAT3 was found increased and phosphorylated in the tumoral region of adenocarcinoma whereas there was no phosphorylation in the tumoral area of squamous carcinoma (Fig. 4 versus f, respectively). These findings are consistent with...
up-regulated activated STAT3 in adenocarcinoma tumour cells as well as with increased levels of IL-6R in Th17 cells in adenocarcinoma as compared to the tumoural region of the squamous carcinoma. Thus, further supporting the possibility to use anti-Th17 immunostrategies to defeat lung adenocarcinoma as well as the presence of an auto-activation of STAT3 in the adenocarcinoma cells.

Loss of correlation between BATF and STAT3 mRNA in the tumoural region of NSCLC. To start to investigate the correlation between BATF and STAT3 mRNA expression and the pathway activated by IL-6 via IL-6R, we analysed the correlation between BATF and STAT3 mRNA in the tumoural and control regions of adenocarcinoma and squamous carcinoma (Fig. 5). Here we found a positive correlation between BATF and STAT3 in the control region of both adenocarcinoma and squamous cell carcinoma. However, this positive correlation was lost in the tumoural region of both but especially in the tumoural region of adenocarcinoma (Fig. 5b, d). Taken together, these data suggest an IL-6R/BATF/STAT3 correlation in tumour free lung tissue.

Table 1 | Clinical data of the patients analysed in this study

| Patient Number | Sample-ID | Histological Classification | Grading | T | N | M | Gender | Age |
|----------------|-----------|----------------------------|---------|---|---|---|--------|-----|
| 1              | P2        | ADC                        | G3       | 3 | 0 | 0 | Male   | 69  |
| 2              | P4        | ADC                        | G3       | 3 | 0 | 0 | Male   | 63  |
| 3              | P5        | ADC                        | G3       | 2b| 0 | 0 | Male   | 71  |
| 4              | P6        | ADC                        | G3       | 2 | 2 | # | Female | 64  |
| 5              | P7        | ADC                        | G3       | 3 | 0 | 0 | Male   | 68  |
| 6              | P12       | ADC                        | G3       | 2b| 1 | 0 | Male   | 70  |
| 7              | P14       | ADC                        | G1       | 4 | 0 | 1 | Male   | 75  |
| 8              | P20       | ADC                        | G3       | 2 | 0 | 0 | Male   | 64  |
| 9              | P22       | ADC                        | G3       | 2a| 1 | 0 | Male   | 69  |
| 10             | P26       | ADC                        | G3       | 3 | 0 | 0 | Male   | 48  |
| 11             | P27       | ADC                        | G2       | 1 | 0 | 0 | Female | 63  |
| 12             | P28       | ADC                        | G2       | 1b| 0 | 0 | Female | 38  |
| 13             | P31       | ADC                        | G2       | 1b| 2 | 0 | Female | 70  |
| 14             | P33       | ADC                        | G2       | 2b| 2 | 0 | Male   | 57  |
| 15             | P37       | ADC                        | G3       | 2a| 0 | 0 | Male   | 51  |
| 16             | P38       | ADC                        | G3       | 1a| 0 | 0 | Female | 37  |
| 17             | 3-MP-IL35 | ADC                        | G3       | 2a| 0 | 0 | Female | 79  |
| 18             | 9-MP-IL35 | ADC                        | G2       | 1b| 2 | 0 | Female | 84  |
| 19             | 15-MP-IL35| ADC                        | G3       | 1b| 0 | 0 | Male   | 63  |
| 20             | 16-MP-IL35| ADC                        | G3       | 0 | 0 | 0 | Female | 70  |
| 21             | 17-MP-IL35| ADC                        | G2       | 2 | 0 | 0 | Male   | 74  |
| 22             | 19-MP-IL35| ADC                        | #        | 2b| 0 | 0 | Female | 55  |
| 23             | 22-MP-IL35| ADC                        | G3       | 2b| 1 | 0 | Male   | 68  |
| 24             | 23-MP-IL35| ADC                        | G2       | 2a| 0 | 0 | Male   | 73  |
| 25             | 26-MP-IL35| ADC                        | G3       | 1a| 0 | 1 | Female | 52  |
| 26             | 27-MP-IL35| ADC                        | G3       | 1a| 0 | 0 | Female | 70  |
| 27             | 28-MP-IL35| ADC                        | G3       | 1a| 0 | 0 | Male   | 76  |
| 28             | 32-MP-IL35| ADC                        | G3       | 2a| 2 | 0 | Female | 60  |
| 29             | 34-MP-IL35| ADC                        | G3       | 1 | 0 | 0 | Female | 51  |
| 30             | 35-MP-IL35| ADC                        | G3       | 1b| 0 | 0 | Female | 72  |
| 31             | P1        | SCC                        | G3       | 2 | 0 | 0 | Female | 66  |
| 32             | P11       | SCC                        | G3       | 2 | 0 | 0 | Female | 68  |
| 33             | P15       | SCC                        | G2       | 1b| 0 | 0 | Male   | 67  |
| 34             | P18       | SCC                        | G3       | 2a| 0 | 0 | Male   | 70  |
| 35             | P19       | SCC                        | G2       | 4 | 1 | 0 | Male   | 48  |
| 36             | P24       | SCC                        | G2       | 2a| 1 | 0 | Male   | 61  |
| 37             | P25       | SCC                        | G3       | 2b| 0 | 0 | Male   | 72  |
| 38             | P29       | SCC                        | G2       | 2a| 0 | 0 | Female | 69  |
| 39             | P35       | SCC                        | G2       | 1a| 0 | 0 | Male   | 45  |
| 40             | 1-MP-IL35 | SCC                        | G3       | 1b| 0 | 0 | Male   | 80  |
| 41             | 2-MP-IL35 | SCC                        | G3       | 1a| 0 | 0 | Male   | 57  |
| 42             | 4-MP-IL35 | SCC                        | G3       | 1b| 1 | 0 | Female | 53  |
| 43             | 5-MP-IL35 | SCC                        | G2       | 3 | 0 | 0 | Female | 67  |
| 44             | 8-MP-IL35 | SCC                        | G3       | 3 | 0 | 0 | Male   | 66  |
| 45             | 13-MP-IL35| SCC                        | G3       | 1b| 0 | 0 | Male   | 69  |
| 46             | 14-MP-IL35| SCC                        | G2       | 1a| 0 | 0 | Female | 58  |
| 47             | 21-MP-IL35| SCC                        | G1       | 1b| 0 | 0 | Male   | 41  |
| 48             | 29-MP-IL35| SCC                        | G3       | 1b| 0 | 0 | Male   | 74  |
| 49             | 30-MP-IL35| SCC                        | G3       | 1a| 0 | 0 | Female | 70  |
| 50             | 36-MP-IL35| SCC                        | G3       | 2a| 1 | 0 | Male   | 74  |

T-extent of primary tumor: 0: No evidence of primary tumor; 1a: Tumor 2 cm or less in greatest dimension; 1b: Tumor more than 2 cm but not more than 3 cm in greatest dimension; 2a: Tumor more than 3 cm but not more than 5 cm in greatest dimension; 2b: Tumor more than 5 cm but not more than 7 cm in greatest dimension; 3: Tumor more than 7 cm; 4: Tumor of any size that invades the mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, carina, or with separate tumor nodules in a different ipsilateral lobe.

N-regional lymph node metastasis: 0: No regional lymph node metastasis; 1: Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension; 2: Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s).

M-peripheral metastasis: 0: No distant metastasis; 1: Distant metastasis.

Histopathological grading: G1: well differentiated; G2: moderately differentiated; G3: Poorly differentiated.

# No information available.
Figure 1 | Decreased IL-6R mRNA expression in the tumoural region of NSCLC. (a) - Quantitative Real-time PCR analysis of IL-6R mRNA expression in the tumoural and control lung area of patients with ADC (adenocarcinoma) and SCC (squamous cell carcinoma) (N (ADCcontrol) = 18; N (ADCtumoural) = 17; N (SCCcontrol) = 14; N (SCCtumoural) = 15). Data are shown as mean values ± s.e.m. using student’s t-test *P < 0.05; **P < 0.01; ***P < 0.001. (b) Flow cytometry staining of murine LL/2-luc-M38 tumour cells with an antibody against IL-6R and an isotype control antibody. (c) - Analysis of IL-6Rα protein expression by Immunohistochemistry (IHC) in the control and tumoural lung region of ADC as well as SCC.

Figure 2 | Decreased expression of IL-6R on lung infiltrating CD4+CD25+Foxp-3+ T-cells in a murine model of lung cancer. (a),(b) - Flow cytometry analysis of IL-6R in the lungs of naive as compared to LL/2-luc-M38 injected wild-type mice (Nnaive = 10, NLL/2 = 11). a-Percentage of IL6R+CD4+ T-cells gated on lymphocytes. b- Percentage of IL-6R+ cells gated on CD4+CD25+Foxp-3+ T-cells. Dot plots depict the gating strategy. Bar charts show the mean percentage of gated cells. Data are shown as mean values ± s.e.m. using student’s t-test ***P < 0.001.
contrast in the tumour region auto-activation mechanisms of STAT3 might be BATF independent.

**Increased RORC expression in the tumoural lung region of patients with adenocarcinoma as compared to those carrying lung squamous carcinoma.** We next investigated whether the increase of BATF in ADC samples compared to SCC samples was accompanied by an increase of other Th17 specific transcription factors in the tumoural region. We found that RORC, a key Th17 transcription factor, was significantly up-regulated, like BATF, in the tumoural region of adenocarcinoma compared to the tumoural region of the squamous carcinoma (Fig. 6). In addition, RORC like BATF was found significantly up-regulated in the control region as compared to the tumoural region of the squamous carcinoma. By contrast, a high and homogeneous distribution of RORC was found in the control and tumoural region of adenocarcinoma patients. In summary, RORC is up-regulated in the tumoural region of adenocarcinoma compared to squamous cell carcinoma consistent with BATF and STAT3 up-regulation in this tumour.

**Anti-IL-6R antibody treatment reduced Th17 and induced T regulatory cells.** Since we found Batf mRNA increased in lung CD4+ T cells isolated from lung adenocarcinoma bearing mice, we then reasoned to block IL-6R signaling to see if the Th17 pathway would be down-regulated in a setting of lung adenocarcinoma. We therefore induced Lewis Lung adenocarcinoma growth in the lungs of mice and treated them intranasally with antibodies against the IL-6R (Fig. 7a). In the lungs of anti-IL-6R antibody treated mice we observed an increased number of CD4+CD25+Foxp3+ T regulatory lymphocytes compared to mice treated with IgG control antibodies (Fig. 7b), consistent with an inhibitory role of IL-6 for FOXP-3+CD4+ T regulatory cell development. In affimetrox RNA gene arrays on CD4+ T cells isolated from the lungs of anti-IL-6R antibody treated mice, we observed a significant up-regulation of a series of immunosuppressive cytokines, such as Il10, Il6 and Il21 and of other genes known to be associated with regulatory cells like Folr4, Cita4, Icos, Il2ra and Lag3 (Fig. 7c, d). By contrast, we detected a reduction of IL-17a expression. (Fig. 7e). Intranasal anti IL-6R antibody treatment significantly reduced also Rorc mRNA expression, encoding RORγT, a Th17 specific transcription factor in tumor-infiltrating lung CD4+ T cells both, by real time PCR and by RNA gene array on sorted lung CD4+ T cells, compared to IgG treatment (Fig. 7d–f). Batf did not show up either in the gene array analysis nor was found up-regulated by anti-IL-6R antibody treatment after real time PCR analysis (Fig. 7g). Taking together, these array data demonstrate an inhibitory function of IL-6 on T regulatory cell genes while favouring Th17 differentiation in a setting of adenocarcinoma. These data are consistent with our data on human samples.
Discussion

Here we found increased expression of IL6R, RORC and BATF, a Th17 lineage specific transcription factors in the tumoral region of lung tissue from patients with lung adenocarcinoma as compared to the tumoral region of lung tissue derived from patients with lung squamous cell carcinoma. Similarly, in the tumoral region of adenocarcinoma STAT3, a transcription factor downstream of IL-6R, was found to be increased and was activated via phosphorylation and autophosphorylation. By contrast, in the tumoral region of squamous carcinoma STAT3 was found inactivated and not phosphorylated indicating the lack of activation of this pathway in lung squamous cell carcinoma. This is a big difference and needs further attention in the design of new therapies against different kinds of non-small-cell lung cancer (NSCLC). In fact, squamous cell carcinomas usually develop in smokers, whereas lung adenocarcinoma is typical for non-smokers and thus more linked to genetic mutations.

To start to understand the distribution of IL-6R and its downstream signaling in lung tumour, we first analysed IL-6R expression in an adenocarcinoma cell line used to induce tumour. We could not find IL-6R expression here, consistent with the findings in humans showing increased IL-6R staining in the tumour free control regions of the lung in patients with NSCLC. Moreover, we described in this study down-regulation of IL-6R on the surface of CD4+CD25+FOXP-3+ T regulatory cells infiltrating the lung in experimental adenocarcinoma, indicating a new mechanism by which the adenocarcinoma tumour cells down-regulate IL-6R in T regulatory cells thus allowing local immunosuppression. BATF has been shown to be induced by STAT3 in mouse myeloid leukemic cells. Dominant negative form of STAT3 resulted in lower expression of BATF11,12. Thus, the increased auto-phosphorylation of STAT3 in the tumoral region of adenocarcinoma might be part of a mutational linkage responsible for the up-regulation of BATF in this region both in tumour and in T cells surrounding the tumour. The exact cellular distribution of STAT3/BATF expression in the microenvironment and in the tumour cell itself needs further studies.

However, we found here a positive correlation between BATF and STAT3 in the control region and the loss of this correlation in the tumoral region. This indicates that in the tumoral region STAT3 expression is not dependent on BATF and vice versa. Batf-/- mice were shown to have normally differentiated Th1 and Th2 cells, but lacked
differentiated Th17 cells. When stimulated towards a Th17 lineage in vivo, Batf−/− T cells produced normal levels of IL-2, IFN-γ, and IL-10, but significantly reduced levels of IL-17. STAT3 and BATF have been described to bind to the IL-17A promoter, thus reinforcing the role of RORcTi in this pathway9,10,15.

In a murine model of adenocarcinoma we could demonstrate by using affimetix array that intranasal delivery of anti-IL-6R antibody but not IgG induced T regulatory immunosuppressive genes, such as Il2ra, Ctlar4, Icos, Fosl4, Lag3, Il10, Il21 and Foxp3. Thus, IL-6 pathway inhibits T-regulatory cells and induces RORcTi in lung tumour. Downstream of IL-6R cytokine receptor STAT3 is increased and phosphorylated in the tumoral region derived from adenocarcinoma bearing tissues13. Interestingly, none of the tumoral regions from patients with squamous carcinoma had activated pSTAT3, as shown by western blot analysis. This is consistent with the reduced IL6R, BATF, RORc expression in the squamous cell lung carcinoma.

Autophosphorylation of pSTAT3 favours mutations in this tumour. RORcTi is also activated by pSTAT3. BATF, downstream of pSTAT3 favours IL-10 and IL-17A with RORcTi. This IL-6R-BATF-STAT3-RORcTi axis seems an independently activated pathway in lung adenocarcinoma and not in squamous carcinoma. These findings have implication for the differential treatment of different histological types of lung tumour.

STAT3 is constitutively activated in different lung tumour cell lines and has been described to induce Th17 transcription factors.

Figure 5 | STAT3 mRNA level positively correlates with BATF mRNA expression in the control lung area of NSCLC. Correlation between BATF and STAT3 mRNA expression in the control (a) and tumoural (b) lung area of patients with ADC as well as in the control (c) and tumoural (d) lung region of patients with SCC (N (ADCcontrol) = 16; N (ADCtumoural) = 16; N (SCCcontrol) = 17; N (SCCtumoural) = 17).

Figure 6 | RORc is reduced in the tumoural regions of SCC. Quantitative Real-time PCR analysis of RORc mRNA expression in the tumoural and control lung area of patients with ADC and SCC (N (ADCcontrol) = 22; N (ADCtumoural) = 21; N (SCCcontrol) = 13; N (SCCtumoural) = 13). Data are shown as mean values ± s.e.m. using student’s t-test *P<0.05.

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such as BATF and RORγT. However, IL-6 also inhibits the immunosuppressive marker FOXP-3 explaining why anti IL-6R antibody treatment would not be effective in adenocarcinoma. Thus, it is possible that a better target than IL-6R would be targeting the BATF activity in lung adenocarcinoma, because it could result in a blockade of the immune evasion activated by the adenocarcinoma because this transcription factor induces the immunosuppressive cytokine IL-10 in addition to IL-17A. Moreover, we have described recently that BATF deficient mice have reduced STAT5 and FOXP-3 regulatory cells. Since STAT5 deficient mice have less FOXP-3 regulatory T cells, targeting BATF would have many advantages over blockade of IL-6R antibody or untreated. The fold-change of the mRNA expression of significantly altered genes is shown as a heatmap (c) and as a bar chart (d) (N = 3 per group). (e),(f),(g) - Quantitative Real-time PCR analysis of Il17 (e), Rorc (f) and Batf (g) mRNA expression in CD4+ T-cells, cultured with anti-CD3 and anti-CD28 antibody. CD4+ T-cells were isolated from the lungs of naive or tumour bearing wild-type mice injected with LL2-luc-M38 cells and treated with anti-IL6R antibody or IgG1 isotype control compared to untreated mice. Data are shown as mean values ± s.e.m. Student’s t-test was used. * P<0.05 (N = 10–11 per group).

**Methods**

All the methods described in this manuscript were carried out in accordance with the approved guidelines. Moreover, all the human studies and their experimental protocols were approved by the local ethics review board of the University of Erlangen (Re-No: 56_12B) and registered at DRKS-ID: DRKS00005376 available online. The experiment protocols performed in this study in mice were approved by the "Regierung Mittelfranken" in the "Asthma und Lungentumormodelle" file number:Az. 54.2532.1-2/10.

**Human subjects and study population.** The study was performed at the University of Erlangen in Germany in agreement with the local ethics review board of the University of Erlangen. Patients’ confidentiality was maintained and informed consent was obtained from each patient. The histological types of lung cancer were classified according to the histological classification of the World Health Organization (WHO), formulated in 2004. The staging of lung cancer was based on the Cancer TNM Staging Manual, formulated by the International Association for the Study of Lung Cancer (IASLC) in 2010. As shown in Table 1, this study was done by analyzing samples stored frozen immediately after thoracic surgery was performed and transported to the adjacent Pathology Department at the University of Erlangen (CCG-Bank). From the pathology, the remaining material was immediately sectioned to divide the tumoural area from the peritumoural (2 cm surrounding the tumoural region) and the control region taken at least 5 cm far from the tumoural region (Fig. S1). Immediately after, the lung pieces were transported on ice to the department of Molecular Pneumology at the University of Erlangen and there stored frozen immediately. This study is registered at the "Deutsches Register Klinischer Studien". Registration number DRK S00005376.
Quantitative Real-Time PCR (qPCR) human. Total RNA was extracted from frozen tissue samples using peqGold RNA Pure (peqlab, Erlangen, Germany) according to manufacturer's instructions. 1 µg of the resulting RNA was reverse-transcribed into cDNA via the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to manufacturer's protocol. Each qPCR reaction mix contained 15 ng of cDNA, 300 nM transcript-specific forward and reverse primer and 1x SYBR Green Supermix (Bio-Rad, Munich, Germany) in a total volume of 20 µL. qPCR primers were purchased from Eurofins-MWG-Operon, Ebersberg, Germany. The primer sequences are given for HPRT (fw: 5′-TGA CAG TGG CAA AAC AAT GCA-3′; rev: 5′-GGT CCT TTT CAG CAA GGT-3′), BATF (fw: 5′-AGC GAAG CAG CTG GAG AAA CA-3′; rev: 5′-TTC AGC ACC GAC GTG AAG TA-3′), IL6R (fw: 5′-GAC AAT GGC ACT GTC GTG TC-3′; rev: 5′-GCT AAT TGG CAG GAG AAC TT-3′), ROCT (fw: 5′-TGA GAA CAG GGA GCC AA-3′; rev: 5′-CCA CAG ATT TTT CAA GGA ATT A-3′), STAT3 (fw: 5′-TTT GTG ATC GAG GGA CG-3′; rev: 5′-GCT GCA ACT CCT CAA GTT TC-3′). Reactions were performed for 50 cycles with an initial activation for 2 minutes at 98°C, followed by 30 cycles at 95°C and hybridization and elongation for 10 min at 60°C. QPCR reactions were performed using the CFX-96 Real-Time PCR Detection System (BIO-RAD, Munich, Germany), and analysed via the CFX Manager Software. The relative expression level of specific transcripts was calculated with respect to the internal standard, HPRT. Expression levels were normalized to Control lung tissues using AACT calculation.

BATF Immunohistochemistry. Immunohistochemistry for BATF was performed in collaboration with the Institute of Anatomy at the University of Erlangen on 6 µm thick cryosections from lung tissue taken from the tumoral or control lung area (at least 5 mm away from tumour regions) of patients with adenocarcinoma and squamous cell carcinoma. The sections were stored frozen until staining was performed. The sections were fixed for 5 minutes with 4% paraformaldehyde in PBS at room temperature followed by three washes 10 minutes each in PBS. To avoid non-specific staining lung tissue sections were incubated in Biotin (InVitrogen) for 1 hour. The primary antibody, anti-human BATF made in rabbit (D7CS, Cell Signaling Technology) was then applied (working dilution 1:200 in PBS) to the tissue sections overnight at 4°C in a humid incubation chamber. Next day, sections were washed three times in PBS. Slides were then incubated for additional 30 minutes in PBS under shaking conditions. At this point the sections were washed with 3 washes in PBS and dried with a KimWipe. The sections were then incubated for 20 minutes in 5% milk powder and 0.1% Tween-20 in PBS and incubated overnight at room temperature. The next day the sections were washed 3 times in PBS (0.1%Tween20) for 5 min each. The sections were then incubated with the HRP-conjugated secondary antibody, soaked overnight in a humid incubation chamber at 4°C. Sections were washed twice with PBS (0.1% Tween20) and once with PBS, for 5 min each. For detection of the proteins the sections were washed with three times PBS (0.1%Tween20) for 5 min each.

Protein isolation and western blot analyses. Lung tissue samples were lysed using protein extraction buffer (50 mM Tris, 0.2 M NaCl, 2 mM EDTA, Complete Mini EDTA free (Roche Diagnostics, Mannheim, Germany)), homogenized and sonicated in an ultrasonic bath for 2 min., followed by 15 min. of incubation on ice and centrifugation (30 min., 4°C, 12000 rpm). The supernatant containing the proteins was collected and protein concentration was determined via Bradford assay. Each western blot sample contained 30–50 µg proteins and was then loaded (4% for Biot-Roll, Roth, Karlsruhe, Germany) in a total volume of 20 µL. Samples were heated at 95 °C for 5 min. and subsequently cooled on ice prior to loading onto 10% polyacrylamide gel. Polyacrylamide gel electrophoresis was performed at 80 V for 1.5 h. Proteins were then transferred to a nitrocellulose membrane (PAGE Ex Gel, Lonza, Rockland, USA) at 200 mA for 45 min. The membrane was washed with 0.1% Tween-20 in PBS for 0.05% Tween-20 at room temperature for 1 h. After incubation in blocking buffer (5% milk powder, 0.1% Tween20 in PBS) at room temperature, the primary antibody was applied and incubated over night at 4°C. The next day the membrane was washed 3 times in PBS (0.1%Tween20) for 5 min each. The membrane was incubated with the HRP-conjugated secondary antibody, soaked overnight in PBS (0.1% Tween20) at room temperature (5°C) followed by a second wash, and subsequently incubated in a solution containing 5 µg/mL DAB (4′,7-diamino-2-phenylindol-dihydrochloride; Boehringer, Mannheim) by embedding the sections in DAPI-glycerol solution (PBS-glycerol 1:1, adding 10 µl of 2 mg/ml DAPI stock solution). The sections were then examined with a Keyence Biozero BZ9000 microscope (Keyence Deutschland, Neu-Schöneberg, Germany).

IL-6R alpha Immunohistochemistry. The Immunohistochemistry (IHC) for IL-6R was performed as described above for BATF-IHC with the following modifications. The primary antibody, mouse monoclonal anti-human IL-6R (Invitrogen) was applied to the tissue sections overnight at 4°C in a humid incubation chamber at the working dilution 1:50 in PBS.

Next day, sections were washed three times in PBS and then incubated for additional 30 minutes in PBS under shaking conditions. At this point the secondary antibody, goat anti rabbit Alexa 488 (Invitrogen) (1:200 in PBS) was applied in the dark for 1 hour. The residual secondary antibody was washed out with three washes with PBS.

The cell nuclei were then stained with DAPI (4′,6-diamidino-2-phenylindol-dihydrochloride; Boehringer, Mannheim) by embedding the sections in DAPI-glycerol solution (PBS-glycerol 1:1, adding 10 µl of 2 mg/ml DAPI stock solution). The slides were then examined with a Keyence Biozero BZ9000 microscope (Keyence Deutschland, Neu-Schöneberg, Germany).

Flow cytometry staining. Flow cytometry analyses were performed with 1 × 10^6 total cells per sample and staining. The cells were washed with PBS, followed by incubation (30 min., 4°C, dark) with the respective mix of antibodies against surface proteins, solved in PBS (ad 80 µl). After an additional washing step the cells were fixed and permeabilized with Fixation/Permeabilization solutions according to manufacturer's protocol (eBioscience) and incubated (30 min., 4°C, dark) with antibodies against intracellular proteins, solved in Permeabilization buffer (eBioscience) (ad 80 µl). After a final washing step with Permeabilization buffer (eBioscience) the cells were resuspended in PBS+ EDTA (Lonza). For FACS analyses of surface markers alone, the cells were resuspended in PBS+ EDTA followed by incubation with the surface antibodies. No fixation and permeabilization was performed in that case. The subsequent flow cytometry measurements were performed via FACS Calibur and analyzed using CellQuest Pro version 4.0.2 (BD Biosciences, Heidelberg, Germany). The following antibodies were used during this study. Mouse anti-mouse CD4 FITC (BD Bioscience, Heidelberg, Germany), Anti-mouse CD25 PerCP (BD Bioscience, Heidelberg, Germany), Anti-mouse IL6R (eBioscience, Frankfurt, Anti-human/mouse FOXP3 APC (Miltenyi Biotec, Bergisch-Gladbach, Germany).

CD4+ T-cell isolation. Single cell suspensions from murine lungs were prepared as previously described[15]. Isolation of CD4+ T-cells from total lung cells was performed using anti-mouse CD4 MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) by positive selection according to manufacturer’s protocol. CD4+ T-cells were cultured in RPMI medium, supplemented with 10% FCS and 1% Pen/Strep, at 37°C and 5% CO2. At day 9 and 12 after tumour induction mice were treated with 90 µg of zL6R antibody (Chugai Pharmaceuticals, Japan) or IgG1 Isotype control (R&D Systems, Minneapolis, USA), resolved in 45 µl of PBS, intranasally (i.n.). Mice were sacrificed at day 15 after tumour cell injection. Lungs were removed and analysed as described below.

Flow cytometry staining. Flow cytometry analyses were performed with 1 × 10^6 total cells per sample and staining. The cells were washed with PBS, followed by incubation (30 min., 4°C, dark) with the respective mix of antibodies against surface proteins, solved in PBS (ad 80 µl). After an additional washing step the cells were fixed and permeabilized with Fixation/Permeabilization solutions according to manufacturer’s protocol (eBioscience) and incubated (30 min., 4°C, dark) with antibodies against intracellular proteins, solved in Permeabilization buffer (eBioscience) (ad 80 µl). After a final washing step with Permeabilization buffer (eBioscience) the cells were resuspended in PBS+ EDTA (Lonza). For FACS analyses of surface markers alone, the cells were resuspended in PBS+ EDTA followed by incubation with the surface antibodies. No fixation and permeabilization was performed in that case. The subsequent flow cytometry measurements were performed via FACS Calibur and analyzed using CellQuest Pro version 4.0.2 (BD Biosciences, Heidelberg, Germany). The following antibodies were used during this study. Mouse anti-mouse CD4 FITC (BD Bioscience, Heidelberg, Germany), Anti-mouse CD25 PerCP (BD Bioscience, Heidelberg, Germany), Anti-mouse IL6R (eBioscience, Frankfurt, Anti-human/mouse FOXP3 APC (Miltenyi Biotec, Bergisch-Gladbach, Germany).

Quantitative Real-Time PCR (qPCR) in mouse samples. Total RNA was extracted from lung derived CD4+ T-cells, using peqGold RNA Pure (peqlab, Erlangen, Germany) according to manufacturer’s instructions. 1 µg of the resulting RNA was reverse-transcribed into cDNA via the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to manufacturer’s protocol. Each qPCR reaction mix contained 15 ng of cDNA, 300 nM transcript-specific forward and reverse primer and 2x SytoFast™ Green Supermix (BIO-RAD, Munich, Germany) in a total volume of 20 µl. qPCR primers were purchased from Eurofins-MWG-Operon, Ebersberg, Germany. Each qPCR reaction mix contained 15 ng of cDNA, 300 nM transcript-specific forward and reverse primer and 2x SytoFast™ Green Supermix (BIO-RAD, Munich, Germany) in a total volume of 20 µl. qPCR primers were purchased from Eurofins-MWG-Operon, Ebersberg, Germany. Each qPCR reaction mix contained 15 ng of cDNA, 300 nM transcript-specific forward and reverse primer and 2x SytoFast™ Green Supermix (BIO-RAD, Munich, Germany) in a total volume of 20 µl. qPCR primers were purchased from Eurofins-MWG-Operon, Ebersberg, Germany.
Gene Array. In collaboration with the Institute of Human Genetics at the University of Erlangen (prof A. Ekici) a gene array was performed. Briefly, RNA was isolated from lung CD4+ T-cells of LL2-luc-M38 injected wild-type mice that were either untreated, treated with anti-IL6R antibody or with IgG1 isotype control as described above. After quality control analysis of the isolated RNA, the GeneChip Gene 1.0 ST Array (Affymetrix, Santa Clara, USA) was used for further analysis.

Statistical analysis. Differences were evaluated for significance (P<0.05) by the unpaired student’s t-test using the GraphPad PRISM software. Data are given as mean values ± s.m.

Acknowledgments
The authors thank Sonja Trump, Adriana Geige at the Molecular Pneumology department at the University of Erlangen for their technical support. The authors are also grateful to Prof. Arif Ekici and his team especially Dr. Fulvia Ferrazzi from the Institute of Human Genetic at the University of Erlangen, in Erlangen for performing and analysing with us the Gene Arrays. This work was supported by the IZKF grant A59 and by the Molecular Pneumology Department in Erlangen.

Author contributions
S.F. designed the experiments and L.B., K.A., N.B. performed the experiments presented in this study. D.I.T., T.R., H.S. and A.H. are responsible for the clinical part of this study. Martin Schicht and L.B. performed and analysed IHC for BATF and IL-6R. S.F. supervised the research, analysed the data and wrote the manuscript.

Additional information
The present work was performed in fulfilment of the requirements for obtaining the degree “Dr. med.” of Liubov Balabko at the Friedrich-Alexander-University Erlangen-Nürnberg (FAU), Erlangen.

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

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

How to cite this article: Balabko, L. et al. Increased expression of the Th17-IL-6R/pSTAT3/BATF/IFN-γ T-axis in the tumoural region of adenocarcinoma as compared to squamous cell carcinoma of the lung. Sci. Rep. 4, 7396; DOI:10.1038/srep07396 (2014).

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