STAT3 expression by myeloid cells is detrimental for the T-cell-mediated control of infection with *Mycobacterium tuberculosis*

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Abstract

STAT3 is a master regulator of the immune responses. Here we show that *M. tuberculosis*-infected *stat3*<sup>fl/fl</sup> <sup>lysm cre</sup> mice, defective for STAT3 in myeloid cells, contained lower bacterial load in lungs and spleens, reduced granuloma extension but higher levels of pulmonary neutrophils. STAT3-deficient macrophages showed no improved control of intracellular mycobacterial growth. Instead, protection associated to elevated ability of *stat3*<sup>fl/fl</sup> <sup>lysm cre</sup> antigen-presenting cells (APCs) to release IL-6 and IL-23 and to stimulate IL-17 secretion by mycobacteria-specific T cells. The increased IL-17 secretion accounted for the improved control of infection since neutralization of IL-17 receptor A in *stat3*<sup>fl/fl</sup> <sup>lysm cre</sup> mice hampered bacterial control. APCs lacking SOCS3, which inhibits STAT3 activation via several cytokine receptors, were poor inducers of priming and of the IL-17 production by mycobacteria-specific T cells. In agreement, *socs3*<sup>fl/fl</sup> <sup>cd11c cre</sup> mice deficient of SOCS3 in DCs showed increased susceptibility to *M. tuberculosis* infection. While STAT3 in APCs hampered IL-17 responses, STAT3 in mycobacteria-specific T cells was critical for IL-17 secretion, while SOCS3 in T cells impeded IL-17 secretion. Altogether, STAT3 signalling in myeloid cells is deleterious in the control of infection with *M. tuberculosis*.

Author summary

We studied the role of STAT3, a major regulator of immunity, in the control of the infection with *M. tuberculosis*. *Stat3*<sup>0/0</sup> <sup>lysm cre</sup> mice, deficient in STAT3 in myeloid cells, showed lower bacterial levels in organs and reduced extension of lung granulomas after infection with *M. tuberculosis*. STAT3-deficient APCs stimulated with innate receptor agonists released high levels of IL-6 and IL-23, and promoted IL-17 production by mycobacteria-specific CD4+ T cells. Increased IL-17 levels accounted for the increased resistance to *M. tuberculosis* of the STAT3-deficient mice. Instead, *stat3*<sup>0/0</sup> <sup>lysm cre</sup> macrophages showed no improved control of mycobacterial growth. SOCS3 is a negative regulator of STAT3 activation. The ability of *socs3*<sup>0/0</sup> <sup>lysm cre</sup> APCs to secrete IL-6 and IL-
23 and to stimulate IL-17 production by antigen-specific T cells was reduced. In agreement, mice lacking SOCS3 in DCs showed increased susceptibility to *M. tuberculosis* infection. Different to a role in myeloid cells, STAT3 expression by mycobacteria-specific T cells was required for IL-17 secretion while SOCS3 in T cells hampered IL-17 production. Therefore, despite STAT3 expression in T cells is required for Th17 differentiation, STAT3 in APCs hampers secretion of Th17 promoting cytokines and the secretion of IL-17 by mycobacteria-specific T cells and reduces the resistance of mice to infection with *M. tuberculosis*.

**Introduction**

Tuberculosis (TB), caused by infection with *Mycobacterium tuberculosis*, remains a leading public health problem worldwide. TB causes 9 million new cases and 1.5 million deaths each year [1]. However, host factors determining the outcome of infection are not completely understood.

A host counters mycobacterial infections primarily via Th1 immune responses that involve cellular effector mechanisms such as macrophage activation [2, 3]. IL-12 secreted by APCs is crucial for the differentiation and maintenance of IFN-γ-secreting antigen-specific Th1 cells [4, 5] and both IL-12 and IFN-γ mediate mycobacterial control in mice and man [6–9].

The transcription factor STAT3 is a central regulator of immunity, mediating inflammatory but also anti-inflammatory responses [10, 11]. The functions of STAT3 are pleiotropic. STAT3 is activated by phosphorylation in response to cytokines of the IFN-receptor family (such as IL-10) and by some members of the IL-2 receptor family that uses the common γ chain receptor or after stimulation of several receptor tyrosine kinases (EGF, CSF-1, and PDGF). Additionally, STAT3 is activated by the common signal transducing molecule gp130 utilized by the IL-6 receptor family [12], and in response to G-CSF and leptin as their receptors are homologous to gp130.

STAT3 is critical for defense against bacterial and fungal infections. Low IL-17 secreting T-cell proportions were reported in patients bearing STAT3 mutations. These patients were prone to chronic candidiasis and staphylococcal diseases [13]. Chronic candidiasis is frequently present in patients deficient in IL-17 receptor A [14]. STAT3 deficient patients may also display impaired immunity against chronic viral infections [15, 16].

In mice, knockout of STAT3 is lethal, so *in vivo* studies on STAT3 functions have been performed using conditional knock out mice. Stat3<sup>fl/fl</sup> lysm cre mice, deficient in STAT3 in myeloid cells, display enhanced susceptibility to endotoxic shock and develop chronic enterocolitis with age [17]. The phenotype of these animals is similar to IL-10<sup>−/−</sup> mice, including increased expression of TNF and other inflammatory cytokines, since IL-10 suppresses induction of TNF-α via STAT3 [18]. Recently, STAT3 was shown to favour intracellular growth of *M. tuberculosis* in human macrophages [19]. Moreover, the presence of pSTAT3+ monocytes associated with the progression of the disease in *M. tuberculosis* infected non-human primates [20].

We have previously analysed the role of SOCS3, a molecule that inhibits STAT3 activation after triggering of several cytokine and growth factor receptors, and found that mice devoid in SOCS3 in myeloid or lymphoid cells showed increased susceptibility to *M. tuberculosis* [21].

The role of STAT3 during infection with *M. tuberculosis* in vivo is still unknown. We here examine the role of STAT3 in *M. tuberculosis* by using stat3<sup>fl/fl</sup> lysm cre mice. We highlight that STAT3 expression in APCs inhibits Th17 associated responses resulting in an increased susceptibility to infection with *M. tuberculosis*.
Results

Stat3<sup>0/0</sup> lysm cre mice are resistant to infection with <i>M. tuberculosis</i>

First, the role of STAT3 expression in myeloid cells in the control of infection with <i>M. tuberculosis</i> was examined using stat3<sup>0/0</sup> lysm cre mice. Lungs and spleens from stat3<sup>0/0</sup> lysm cre mice after 4 and 8 weeks of infection showed significantly lower <i>M. tuberculosis</i> burden than stat3<sup>0/0</sup> littermates (Fig 1A and 1B). A smaller area of the lung parenchyma of stat3<sup>0/0</sup> lysm cre mice was occupied by granulomas when compared to control lungs 4 but not at 8 weeks after infection (Fig 1C).

The density of granulocytes in the lung parenchyma was determined either by H&E staining of sections (Fig 1D) or by labelling of CD11b<sup>+</sup>CD11c<sup>-</sup>Ly6C<sup>dimm</sup>Ly6G<sup>+</sup> neutrophils (Fig 1E and 1F) in lung suspensions from stat3<sup>0/0</sup> lysm cre and stat3<sup>0/0</sup> mice 3 and 4 weeks after <i>M. tuberculosis</i> infection. The neutrophil density (Fig 1D–1F) and the levels of neutrophil myeloperoxidase (mpo) and elastase (elane) mRNAs (Fig 1G and 1H) were also higher in lungs from stat3<sup>0/0</sup> lysm cre mice at 4 and 8 but not at 14 weeks after infection compared to controls (Fig 1D–1H and S1A–S1C Fig).

Stat3-deficient and control BMM show similar control of the growth of intracellular <i>M. tuberculosis</i>

Activated STAT3 hampers TNF expression [22, 23]. Lungs from stat3<sup>0/0</sup> lysm cre mice infected with <i>M. tuberculosis</i> (Fig 2A) as well as BMM infected with <i>M. tuberculosis</i> or BCG (Fig 2B–2D) showed higher TNF protein and mRNA levels than controls. Since TNF has been shown to mediate <i>M. tuberculosis</i> control in macrophages [24], we speculated that stat3<sup>0/0</sup> lysm cre macrophages could display a better control of intracellular mycobacteria.

A higher frequency of stat3<sup>0/0</sup> lysm cre BMMs were infected when measured 4 h after co-incubation with the <i>M. tuberculosis</i>, although the number of bacteria per infected cell was similar (Fig 2E–2G). Three days after infection <i>M. tuberculosis</i> infected mutant and WT BMM showed similar numbers of infected cells and bacteria per total or infected cell (Fig 2E–2G). Stat3<sup>0/0</sup> lysm cre BMM showed no improved control of <i>M. tuberculosis</i> or BCG growth in vitro 6 days after infection as measured by CFU in lysates (Fig 2H and 2I). Altogether, we observed no indication of an improved bacterial growth control or reduced bacterial uptake in stat3<sup>0/0</sup> lysm cre BMM.

Role of STAT3 and SOCS3 in APCs in the regulation of T cell priming

Several cytokines controlled by STAT3 are potent regulators of the expression of co-stimulatory molecules on APCs. Therefore, we studied whether STAT3 played a role in regulation of T cell priming. As expected, the density of co-stimulatory molecules CD80 and CD86 as well as MHC-II levels increased on BMDCs after mycobacterial stimulation. The expression of MHCII, CD80 and CD86 in either control or stat3<sup>0/0</sup> lysm cre BMDCs before or after mycobacterial stimulation was similar (Fig 3A–3C). To investigate if the expression of STAT3 by myeloid cells could modulate T cell priming during infection with <i>M. tuberculosis</i> T cell receptor transgenic T cells specific for the immunodominant mycobacterial Ag85B<sub>240-254</sub> peptide (p25-tg) cells were inoculated i.v. into stat3<sup>0/0</sup> lysm cre or stat3<sup>0/0</sup> mice 17 days after infection with <i>M. tuberculosis</i> (Fig 3D). Three days after transfer, the expression of CD69 (which increases after T cell receptor triggering) and CD62L (the L-selectin ligand that hampers T cells to traffic to the periphery) was measured on p25-tg T cells and host T cells from the mediastinal lymph nodes (MLN). The expression of CD69 was increased and the expression of CD62L was reduced in p25-tg T cells from MLN of infected mice when compared to
Fig 1. *Stat3*fl/fl lysm cre mice are resistant to infection with *M. tuberculosis*. *Stat3*fl/fl lysm cre and *stat3*fl/fl littermate controls were sacrificed at indicated time points after aerosol infection with *M. tuberculosis* and colony forming units (CFU) per lung (A) and spleen (B) were assessed. The CFU per organ of individual mice and the median per group at the indicated time points after infection are depicted. For each time point 8–10 control and 8–10 mutant mice were infected simultaneously. We performed separated experiments for 4 and 8 weeks post infection. Only one representative of the two experiments for 4 as well as 8 weeks post infection is depicted. Differences in CFU are significant (*p*<0.05, **p**<0.01, ***p***<0.001 Mann Whitney U test). Histopathological
scoring of hematoxylin-eosin stained paraffin lung sections from stat30/0; lym cre and stat30/0; mice measured 4 and 8 weeks after infection with M. tuberculosis. The mean ± SEM % lung area with granulomas (C) and the relative neutrophil density score (D) are depicted. Differences are significant (*p<0.05, **p<0.001 Student's t test). A representative dot plot (E) and the frequency (F) of CD11b+CD11c Ly6C+Ly6G- neutrophils in lungs stat30/0; lym cre and stat30/0; mice 3 and 4 weeks after infection with M. tuberculosis are shown. Differences between 4 mice at each time point are significant (*p<0.05 Student's t test). Total RNA was extracted from the lungs of stat30/0; lym cre and stat30/0; mice at the indicated time points after infection with M. tuberculosis. The relative concentration of neutrophil elastase (elane) (G) and myeloperoxidase (mpo) transcripts (H) in relation to hpgr mRNA levels in the same sample was determined by real time PCR. The mean fold induction of these transcripts ± SEM is depicted. Differences with control mice are significant (*p<0.05, **p<0.01 Student-t test).

https://doi.org/10.1371/journal.ppat.1006809.g001

uninfected control mice. Similar levels of the CD69 and CD62L were expressed by p25-tg or host T cells from stat30/0; lym cre or stat30/0; infected mice (Fig 3E and 3F).

SOCS3 inhibits STAT3 activation by different cytokine receptors, e.g. those of the IL-6 receptor family [10]. In accordance with the results obtained with socs30/0; lym cre mice [21], socs30/0; cd11c cre mice showed higher bacterial levels in lungs and spleens after infection with M. tuberculosis than control animals (Fig 3G and 3H). The cd11c cre transgene has been shown to be expressed in the majority of conventional and plasmacytoid DCs [25].

Mycobacteria-stimulated BMDC from socs30/0; lym cre showed lower levels of MHCII, CD80 and CD86 than control cells (Fig 3I–3L). When T cell priming in vivo was studied by transferring p25-tg naïve T cells into M. tuberculosis infected animals, the density of CD69 on donor p25-tg T cells and on host MLN T cells was lower in WT mice as compared to socs30/0; lym cre mice recipients (Fig 3M and 3O). The p25-tg T cells in the MLN of socs30/0; lym cre mice also showed lower surface density of CD69 as compared to those from WT-infected mice (Fig 3N and 3P). However, the expression of CD69 in host MLN T cells from M. tuberculosis-infected WT and socs30/0; lym cre mice was similar (Fig 3P).

Thus, deficiency of SOCS3 but not STAT3 in APCs regulates T cell priming during M. tuberculosis infection in vivo.

STAT3 in myeloid cells impairs IFN-γ secretion by mycobacteria-specific T cells

IFN-γ is required for protection against M. tuberculosis [2, 3]. STAT3 has been shown to inhibit the transcription of IL-12, a potent inducer of IFN-γ secretion by T cells [23]. We then analysed if the increased resistance to M. tuberculosis of stat30/0; lym cre mice is associated with higher IFN-γ secretion by T cells. Higher levels of IL-12p40 (the α-chain of IL-12 and IL-23) in supernatants and il12p40 mRNA in cell lysates of BCG-infected stat30/0; lym cre BMM or BMDC compared to controls were measured (Fig 4A and 4B). The il12p40 mRNA coding for the β-chain of the IL-12 heterodimer was also expressed in higher amounts by M. tuberculosis- or BCG-stimulated stat30/0; lym cre BMDC compared to controls (Fig 4C).

Thus, whether STAT3-deficient APCs are better stimulators of IFN-γ secretion by mycobacteria-specific T cells than WT APCs was investigated. To test this hypothesis, p25-tg T cells were incubated with either BCG- or M. tuberculosis-infected stat30/0; lym cre or stat30/0; BMDCs and the IFN-γ titers in the supernatants measured. IFN-γ levels in supernatants were elevated compared to those incubated with WT APCs (Fig 4D and 4E). Supernatants from cultures of p25-tg T cells incubated with heat-killed BCG-stimulated stat30/0; BMDC or BMM also contained higher levels of IFN-γ than those using control APCs (Fig 4F), indicating that infection is not required for such responses. In line with this, IFN-γ levels were higher in supernatants from p25-tg T cells co-incubated with stat30/0; lym cre BMDC or BMM stimulated with oligopeptide p25 (amino acids 240–254) from Ag85b, a major immunodominant H2β epitope [26] recognized by the p25-tg T cells, in presence of LPS (Fig 4G).
Confirming previous results [21, 27], socs3^{0/−}/lysm cre BMDC showed diminished IL-12 secretion after mycobacterial stimulation (Fig 4H). Furthermore, IFN-γ secretion by p25-tg T-cells incubated with mycobacteria-infected socs3^{0/−}/lysm cre or socs3^{0/−}/cd11 cre BMDCs was reduced (Fig 4I and 4J).

In line with these results, mycobacteria-infected il12p40^{−/−} BMDCs showed reduced ability to trigger IFN-γ secretion by p25-tg T cells than controls (Fig 4K). Moreover, the addition of
Myeloid STAT3 impairs *M. tuberculosis* control

**A**

|               | Control | Infected |
|---------------|---------|----------|
| *stat3*^+/+^  |         |          |
| *stat3*^−/−^  |         |          |

**B**

|               | Control | Infected |
|---------------|---------|----------|
| *lys*^+/+^    |         |          |
| *lys*^−/−^    |         |          |

**C**

|               | Control | Infected |
|---------------|---------|----------|
| *CD80*^+^     |         |          |
| *CD80*^−^     |         |          |
| *CD86*^+^     |         |          |
| *CD86*^−^     |         |          |

**D**

![Staining of mediastinal lymph nodes](image)

**E**

|               | p25 T cells | Host T cells |
|---------------|-------------|--------------|
| *CD62L*^+^   |             |              |
| *CD62L*^−^   |             |              |

**F**

|               | p25 T cells | Host T cells |
|---------------|-------------|--------------|
| *CD68*^+^     |             |              |
| *CD68*^−^     |             |              |

**G**

|               |          |          |
|---------------|----------|----------|
| CFU per lung  |          |          |
| CFU per spleen|          |          |

**H**

|               |          |          |
|---------------|----------|----------|
| *soxs3*^+/+^  |          |          |
| *soxs3*^−/−^  |          |          |

**I**

|               |          |          |
|---------------|----------|----------|
| *Socs3*^+/+^  |          |          |
| *Socs3*^−/−^  |          |          |

**J**

|               |          |          |
|---------------|----------|----------|
| *soxs3*^+/+^  |          |          |
| *soxs3*^−/−^  |          |          |

**K**

|               |          |          |
|---------------|----------|----------|
| *SOCS3*^+/+^  |          |          |
| *SOCS3*^−/−^  |          |          |

**L**

|               |          |          |
|---------------|----------|----------|
| *CD80*^+^     |          |          |
| *CD80*^−^     |          |          |
| *CD86*^+^     |          |          |
| *CD86*^−^     |          |          |

**M**

|               |          |          |
|---------------|----------|----------|
| P25 T cells   |          | Host T cells |
| *CD62L*^+^   |          |              |
| *CD62L*^−^   |          |              |
| *CD68*^+^    |          |              |
| *CD68*^−^    |          |              |

**N**

|               |          |          |
|---------------|----------|----------|
| p25 T cells   |          | Host T cells |
| *CD62L*^+^   |          |              |
| *CD62L*^−^   |          |              |
| *CD68*^+^    |          |              |
| *CD68*^−^    |          |              |

**O**

|               |          |          |
|---------------|----------|----------|
| p25 T cells   |          | Host cells |
| *CD62L*^+^   |          |              |
| *CD62L*^−^   |          |              |
| *CD69*^+^    |          |              |
| *CD69*^−^    |          |              |

**P**

|               |          |          |
|---------------|----------|----------|
| uninfected control |          |          |

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*PLOS Pathogens* | https://doi.org/10.1371/journal.ppat.1006809 | January 16, 2018
rec IL-12 restored the capacity of socs3<sup>fl/fl</sup> <i>lysm</i> cre BMDC to stimulate IFN-γ secretion by p25-tg T cells (Fig 4L).

Cells derived from gp130<sup>fl/fl</sup> mice, harbouring a mutation that ablates SOCS3 binding to the gp130, show exaggerated gp130-mediated STAT3 responses [28]. Mycobacteria-infected gp130<sup>fl/fl</sup> BMDCs also showed a reduced ability to stimulate IFN-γ secretion p25-tg T cells compared to WT cells (Fig 4M).

The frequency of IFN-γ-secreting mycobacteria-specific T cells in lung cell suspensions from <i>stat3<sup>fl/fl</sup> lym</i> cre and <i>stat3<sup>fl/fl</sup></i> mice 4 and 8 weeks after infection with <i>M. tuberculosis</i> was similar. The frequencies of lymphoid cell populations (S2A Fig) and of PPD- and PMA/ ionomycin-stimulated IFN-γ secreting CD4<sup>+</sup> or CD8<sup>+</sup> cells (Fig 4N and 4O and S2B and S2C Fig) from lungs <i>stat3<sup>fl/fl</sup> lym</i> cre and <i>stat3<sup>fl/fl</sup></i> mice 4 and 8 weeks were also similar. In addition, levels of <i>ifng</i>, and the IFN-γ-regulated <i>inos</i> and <i>cxcl9</i> transcripts were increased in lungs after infection as compared to uninfected controls, but the titers of these transcripts in lungs from <i>stat3<sup>fl/fl</sup> lym</i> cre and <i>stat3<sup>fl/fl</sup></i> infected mice were comparable (Fig 4P–4R).

**Myeloid STAT3 hamper T<sub>H</sub>17 responses during <i>M. tuberculosis</i> infection**

The neutrophil density and the levels of neutrophil transcripts were enhanced in the lungs of <i>M. tuberculosis</i>-infected <i>stat3<sup>fl/fl</sup> lym</i> cre as compared to control mice (Fig 1D–1H). IL-17 has been shown to stimulate granulopoiesis via G-CSF production and to induce the expression of CXC chemokines involved in granulocyte recruitment [29]. Thus, we investigated whether the increased neutrophil levels in lungs from <i>M. tuberculosis</i>-infected <i>stat3<sup>fl/fl</sup> lym</i> cre was associated with augmented T<sub>H</sub>17 responses. The frequency of IL-17-secreting, PPD-stimulated CD4<sup>+</sup> T cells from lungs from <i>stat3<sup>fl/fl</sup> lym</i> cre mice 4 and 8 weeks after infection with <i>M. tuberculosis</i> were elevated when compared to <i>stat3<sup>fl/fl</sup></i> controls (Fig 5A–5C). Instead, the frequency of γδ T cells in lungs and the frequency of IL-17 secreting pulmonary γδ<sup>+</sup> T cells from WT or <i>stat3<sup>fl/fl</sup> lym</i> infected mice was similar (S3A–S3C Fig).

In addition, levels of <i>il17a</i> and <i>il22</i>, transcripts that code for T<sub>H</sub>17 cytokines were higher in lungs from <i>stat3<sup>fl/fl</sup> lym</i> cre mice than in those from littermate controls when measured at 4 and 8 weeks after infection (Fig 5D and 5E). Higher levels of <i>il17</i> mRNA were also observed in <i>stat3<sup>fl/fl</sup> lym</i> cre mice 14 weeks after infection with <i>M. tuberculosis</i>, while the increase of <i>il22</i> mRNA did not reach statistical significance (S4A and S4B Fig). CXCL5 is a neutrophil chemotactic protein stimulated by IL-17 [30]. The level of <i>cxcl5</i> mRNA was increased in lungs from <i>M. tuberculosis</i>-infected <i>stat3<sup>fl/fl</sup> lym</i> cre mice (Fig 5F).

Substantial <i>in vivo</i> data support the notion that IL-6 and IL-23 are required at different stages of T<sub>H</sub>17-cell differentiation [31, 32]. Levels of <i>il6</i> and <i>il23</i> mRNA were elevated in the lungs of <i>M. tuberculosis</i>-infected <i>stat3<sup>fl/fl</sup> lym</i> cre mice when compared to levels in lungs from WT mice (Fig 5G and 5H).
Myeloid STAT3 impairs *M. tuberculosis* control

**Fig 4. STAT3 in myeloid cells impairs IFN-γ secretion by mycobacteria-specific T cells in vitro.** The concentration of IL-12 p40 in supernatants from mycobacteria-infected stat3γ/γ lym cre and stat3γ/γ BMDCs (A) or BMM (B) at different times after incubation were determined by ELISA. The mean IL-12p40 ± SEM pg/ml from triplicate cultures is depicted. Differences with stat3γ/γ BMDCs are significant (**p<0.01, ***p<0.001 Student t test). Total RNA was extracted from stat3γ/γ lym cre and stat3γ/γ BMDC cultures 24 h after *M. tuberculosis* infection. The mean IL-12p35 mRNA levels ± SEM levels measured by real time PCR are depicted (C) (**p<0.01 Student t test). Stat3γ/γ lym cre and stat3γ/γ BMDC were infected with either BCG (D), *M. tuberculosis* (E) or stimulated with heat killed BCG (F) or with LPS and peptide 25 of Ag85b (G) washed and incubated 6 h after with p25-tg CD4+ naïve T cells (at a ratio of 4:1 BMDC) (G). The concentration of IFN-γ in the culture supernatants was measured by ELISA 72 h after co-incubation. The mean IFN-γ ± SEM from triplicate cultures is depicted (**p<0.001 Student t test). The concentration of IL-12p40 in supernatants from mycobacteria-infected socs3γ/γ lym cre and socs3γ/γ BMDCs was determined by ELISA (H). The mean IL-12p40 ± SEM ng/ml from triplicate cultures is depicted (**p<0.01 and ***p<0.001 Student t test). Socs3γ/γ lym cre (I), socs3γ/γ cd11 cre (I) and socs3γ/γ BMDC were infected with BCG and incubated 6 h after with p25-tg T cells. The concentration of IFN-γ in the supernatants was measured by ELISA 24 h after co-incubation. The mean IFN-γ ± SEM from triplicate cultures is depicted (**p<0.01 Student t test). Il12p40-/- (K), gp130-/- (M) and WT BMDC were infected with BCG and co-incubated with p25-tg T cells as described. Mycobacterial-infected socs3γ/γ lym cre and socs3γ/γ were cultured in presence of recombinant IL-12p70 or left untreated (K) and co-incubated with p25Tg-T cells (L). The mean IFN-γ ± SEM in supernatants from triplicate cultures was measured by ELISA (**p<0.01, ***p<0.001 Student t test). The frequency of IFN-γ-secreting cells in PPD-stimulated pulmonary T cells from stat3γ/γ lym cre and stat3γ/γ mice 4 and 8 weeks after infection with *M. tuberculosis* was analysed by ICS (N, O). The mean frequency of IFN-γ-secreting cells in lungs from anti-IL17RA treated or untreated infected mice was measured by ICS (N, O). The mean frequency of IFN-γ-secreting within CD4+ cells (N) and CD8+ (O) ± SEM is displayed (n = 5 per group). The levels of ifng (P), inos (Q) and cxcl9 (R) mRNA in the lungs of stat3γ/γ lym cre and stat3γ/γ mice before and at the indicated time points after aerosol infection with *M. tuberculosis* were determined by real time PCR.

https://doi.org/10.1371/journal.ppat.1006809.g004

T117 cells show a high degree of developmental flexibility, and when exposed to IL-12 or IL-23, they can rapidly acquire effector functions that are normally associated with T117 responses such as IFN-γ production [33]. These IFN-γ and IL-17 secreting cells were shown to be pathogenic in murine models of autoimmune diseases, and were also associated with murine colitis and human IBDS [34]. The majority of PPD or PMA/ionomycin-stimulated IL-17 secreting CD4+ T cells in lungs from *M. tuberculosis*-infected stat3γ/γ lym cre or stat3γ/γ mice were not IFN-γ co-producers (Fig 5I–5K). We next studied whether IL-17 played a role in the increased control of infection of stat3γ/γ lym cre mice. For these experiments, mice were treated with neutralizing IL-17RA mab (M751) before and during infection with *M. tuberculosis*. Similar bacterial levels were found in lungs and spleens from stat3γ/γ lym cre and stat3γ/γ animals treated with anti-IL17RA mAb. As expected, stat3γ/γ lym cre mice from untreated mice showed reduced bacterial numbers in lungs and spleens than those from stat3γ/γ controls (Fig 5L and 5M).

The levels of mpo mRNA was measured in lungs from anti-IL-17RA to control for the IL17RA neutralization. As expected, levels of mpo mRNA were increased in lungs from *M. tuberculosis* infected stat3γ/γ lym cre mice as compared to WT. In contrast, levels of mpo mRNA in lungs from infected or anti-IL17RA treated stat3γ/γ lym cre and stat3γ/γ mice was similar. Lower titters of mpo mRNA were found in stat3γ/γ lym cre infected mice treated with anti-IL-17RA compared to untreated infected controls, while mpo mRNA levels in anti-IL17RA treated or untreated infected stat3γ/γ mice were similar (Fig 5N).

Hence, increased *M. tuberculosis* control during infection of stat3γ/γ lym cre mice is dependent on IL-17, but IL-17 neutralization did not increase the susceptibility to *M. tuberculosis* of mice with normal STAT3 function.

**STAT3 expression in antigen presenting cells inhibit the generation of IL-17 secreting mycobacteria-specific T cells**

The role by which STAT3 in activated APCs regulates IL-17 secretion by specific T cells was then studied. The mRNA levels of il6 and il23p19 were both increased in BMM and BMDCs co-incubated with mycobacteria in vitro (Fig 6A and 6B and S5A Fig). An increased accumulation of il6 and il23p19 mRNA was also observed after stimulation with the TLR agonists LPS, CpG or Pam3K of stat3γ/γ lym cre as compared to stat3γ/γ BMM at 6 and 24 h after stimulation.
Myeloid STAT3 impairs \textit{M. tuberculosis} control.

\textbf{A} \quad \text{stat}^{3\text{fl/fl}} \quad 4 \text{ weeks p.i.}

\textbf{B} \quad \text{stat}^{3\text{fl/fl}} \text{ lsm} \quad 8 \text{ weeks p.i.}

\textbf{C}

\textbf{D}

\textbf{E}

\textbf{F}

\textbf{G}

\textbf{H}

\textbf{I}

\textbf{J}

\textbf{K}

\textbf{L}

\textbf{M}

\textbf{N}

\textbf{P}

\textbf{Q}

\textbf{R}

\textbf{S}

\textbf{T}

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\textbf{Y}

\textbf{Z}
Fig 5. Myeloid cell expression of STAT3 hamper IL-17 secretion by T cells during M. tuberculosis infection. The frequency of IL-17-secreting PPD and PMA and ionomycin (P.I) -stimulated CD4+ pulmonary T cells from stat3fl/fl lymS cre and stat3fl/fl mice 4 (A) and 8 (B) weeks after infection with M. tuberculosis was measured by FACS. A representative graph plot from PPD stimulated lungs at 4 w after infection (C) and the mean percentage of IL-17-secreting CD4+ cells ± SEM (A, B) are displayed (n = 6 per group; "p<0.05, ""p<0.01 and """"p<0.001 Mann Whitney U test). The mean fold increase of il17a (D), il22 (E), cxccl5 (F), il23p19 (G) and il6 (H) mRNA ± SEM was measured by real time PCR in the total RNA from lungs of stat3fl/fl lymS cre and stat3fl/fl mice at different time points after M. tuberculosis infection (n = 8 per group ""p<0.01 Student’s t test). The mean frequency of PPD (I) and PMA and ionomycin (I) -stimulated CD4+ pulmonary T cells from stat3fl/fl lymS cre and stat3fl/fl mice 8 weeks after infection with M. tuberculosis co-stimulating or not IFN-γ was measured by FACS (n = 4 per group, "p<0.05, ""p<0.01 and """"p<0.001 Mann Whitney U test). A representative graph plot from CD3+CD4+ gated PPD and P.I. stimulated IFN-γ and / or IL-17+ lung cells (K) is depicted. The frequency of both IL-17 and IFN-γ and only IL-17+ secreting cells from stat3fl/fl lymS cre is higher as compared to stat3fl/fl controls; the frequency of IL-17+/IFN-γ- is higher than IL-17+/IFN-γ+ cells. This was determined after either PPD or PMI/ ionomycin stimulation (Two-way ANOVA "p<0.05 and """"p<0.0001). Stat3fl/fl lymS cre and stat3fl/fl littermate controls were treated i.p. 1 day before and once per week after aerosol infection with M. tuberculosis with 500 μg anti-IL-17RA M751or left untreated. Mice were sacrificed 4 weeks after the infection and colony forming units (CFU) per lung (L) and spleen (M) were assessed. The CFU per organ of individual mice and the median per group at the indicated time points after infection are depicted. Differences in CFU are significant ("p<0.05, ""p<0.01 Mann Whitney U test). The mean fold increase of il17a mRNA ± SEM was measured by real time PCR in lysates from lungs of stat3fl/fl lymS cre and stat3fl/fl mice 4 weeks after infection with M. tuberculosis treated or not with anti-IL-17RA as described above (n ≥ 4 per group; "p<0.05 Student’s t test) (N).

https://doi.org/10.1371/journal.ppat.1006809.g005

(Fig 6C and 6D and S5B Fig). This indicates that STAT3-mediated inhibition of the expression of IL-6 and IL-23 is not restricted to mycobacterial infection or stimulation with mycobacterial molecules. Supernatants from cultures of mycobacteria-infected stat3fl/fl lymS cre BMM or BMDC co-incubated with naïve p25-tg T cells contained higher titers of IL-17 than those using stat3fl/fl controls (Fig 6E–6G). IL-17 levels were also higher in supernatants from p25-tg T cells co-incubated with stat3fl/fl lymS cre BMDC stimulated with either live or heat-killed BCG, M. tuberculosis or peptide 25 from Ag85b in presence of LPS (Fig 6F–6H).

Whether SOCS3 in mycobacteria-infected APCs also regulated IL-17 secretion by antigen-specific T cells was then measured. We found that mycobacteria-infected socs3fl/fl lymS cre BMM contained lower levels of il6 and il23p19 mRNA than their WT counterparts (Fig 6I and 6J). Moreover, p25-tg T cells incubated with socs3fl/fl lymS cre BMM stimulated either with BCG, M. tuberculosis or the cognate p25 peptide secreted lower levels of IL-17 than those stimulated by WT BMDCs (Fig 6K–6M). Similarly, supernatants from co-cultures of p25-tg T cells with mycobacteria-infected gp130fl/fl BMDC contained higher levels of IL-17 than those using wild type BMDCs (Fig 6N).

We then asked whether STAT3 deficiency regulated the levels of socs3 mRNA transcripts in mycobacteria-infected macrophages. M. tuberculosis-infected stat3fl/fl lymS cre and stat3fl/fl BMMs showed similar levels of socs3 mRNA (Fig 6O).

STAT3 and SOCS3 in T cells show a differential regulation of IL-17 and IFN-γ secretion

Different to the inhibitory role of STAT3 in myeloid cells we here showed, STAT3 expression in T cells has been indicated to be required for T117 cell differentiation in vitro and in vivo [31]. SOCS3, via hyperactivation of STAT3, has been shown to increase IL-17 secretion [35]. In order to compare the role of STAT3 and SOCS3 in T cells and APCs in the regulation of cytokine secretion by T cells, stat3fl/fl lck cre p25-tg and socs3fl/fl lck cre p25-tg mice were generated. The culture supernatants of stat3fl/fl lck cre p25-tg T cells stimulated with BCG-infected or Ag85b peptide-pulsed BMDCs showed low or undetectable levels of IL-17 as compared to controls (lck cre p25-tg T cells) (Fig 7A). Instead IL-17 levels in supernatants from socs3fl/fl lck cre p25-tg T cells co-incubated with BCG or peptide loaded BMDCs were higher than controls (Fig 7C).

The titers of IFN-γ in the supernatants of stat3fl/fl p25-tg T cells incubated with BCG- (but not with Ag85b peptide-) stimulated BMDCs were higher than those incubated with control T cells (Fig 7B). IFN-γ levels in supernatants from mycobacteria or peptide pulsed BMDCs
Myeloid STAT3 impairs \textit{M. tuberculosis} control.

\( \text{stat}^{3\text{+/+}} \)  \( \text{stat}^{3\text{+/+}} \text{ lysm} \)

\( \text{IL-17 (ng/mL)} \)

\( \text{Control} \)  \( \text{Mtb} \)

\( \text{IL-17 (ng/mL)} \)

\( \text{BMM-BMDC} \)  \( \text{BMM-BMDC} \text{ Ag85 pept LPS} \)

\( \text{Fold increase \text{Il}23p19 \text{mRNA}} \)

\( 0 \)  \( 6 \)  \( 24 \)  \( 48 \)

\( \text{LPS} \)  \( \text{CpG} \)  \( \text{Pam3K} \)

\( \text{IL-17 (ng/mL)} \)

\( \text{Control} \)  \( \text{Infected} \)

\( \text{BCG} \)  \( \text{HK-BCG} \)

\( \text{Socs3}\text{+/+} \)  \( \text{Socs3}\text{+/+} \text{ lysm cre} \)

\( \text{Socs3}\text{+/+} \)  \( \text{Socs3}\text{+/+} \text{ lysm} \)

\( \text{Socs3}\text{+/+} \text{ lysm cre} \)  \( \text{WT} \)  \( \text{gp130}\text{-/-} \)

\( \text{IL-17 (ng/mL)} \)

\( \text{Control Peptide} \)  \( \text{Control Infected} \)

\( \text{Fold increase \text{socs3 mRNA}} \)

\( 0 \)  \( 6 \)  \( 12 \)  \( 24 \)
incubated with socs3^{+/+} lck cre p25-tg were instead lower than those using control p25-tg T cells (Fig 7D).

Thus, STAT3 in APCs and T cells, has a dissimilar ability to regulate IL-17 secretion by ag-specific T cells, while SOCS3 in APCs and T cells promote T cell mediated IFN-γ secretion.

Discussion

We report here that stat3^{+/+} lysoM cre mice show reduced M. tuberculosis load in lungs and spleens, indicating that STAT3 expression in myeloid cells is detrimental for the control of infection with M. tuberculosis. Despite reduced area of the lung occupied by granuloma the area with inflammation was not reduced and the numbers of infiltrating pulmonary neutrophils were elevated in stat3^{+/+} lysoM cre mice. Neutrophil accumulation late during infection have been associated with susceptibility to M. tuberculosis, whereas early after infection neutrophils play a protective role and contribute to early priming of T cells in the draining lymph node [36–39].

Although mice lacking STAT3 expression in bone marrow progenitors display peripheral neutrophilia under resting conditions [40], the pathways involved in neutrophil mobilization and response to chemokines during inflammation have been shown to be STAT3 dependent [41, 42]. Thus, we consider it unlikely that the increase in pulmonary neutrophils observed during infection occurs as a direct consequence of STAT3 deficiency in these cells. Rather, increased levels of IL-17 and IL-22, cytokines that stimulate the expression of neutrophil recruiting chemokines [29], might contribute to the accumulation of granulocytes in lungs from M. tuberculosis-infected stat3^{+/+} lysoM cre mice. In agreement with this hypothesis, the frequency of IL-17 secreting mycobacteria-specific CD4^{+} T cells, but not of γδ^{+} T cells, were elevated in the lungs from stat3^{+/+} lysoM cre mice compared to controls.

STAT3 expression in APCs proved to be a major regulator of the expression of cytokines that control T cell differentiation. This was shown in STAT3- and in SOCS3-deficient APC, which display higher levels of activated STAT3 when stimulated with mycobacteria or other innate receptor agonists [21, 43]. Thus, while mycobacteria-infected STAT3 deficient APCs showed an improved ability to trigger IL-17 secretion by antigen-specific T cells, the opposite was observed using socs3^{+/+} lysoM cre or gp130^{+/+} BMDCs or macrophages as APCs.
Fig 7. SOCS3 and STAT3 in antigen-specific T cells are important regulators of IL-17 and IFN-γ secretion. Stat3<sup>fl/fl</sup> lck cre p25-tg T cells (A, B), socs3<sup>fl/fl</sup> lck cre p25-tg T cells (C, D) or control p25-tg T cells were incubated for 3 days with either BCG, peptide 25 and LPS-stimulated or untreated BMDCs. The mean concentration of IL-17 (A, C) and IFN-γ (B, D) ± SEM in supernatants from triplicate cultures is shown (**p<0.01 and ***p<0.001 Student’s t test). Graphical summary (E): Mice deficient in STAT3 in myeloid cells show increased resistance while socs3<sup>fl/fl</sup> lym cre mice displaying augmented STAT3 activation had impaired resistance against infection with <i>M. tuberculosis</i>. The differential control of infection in excess or deficiency of STAT3 is not due to the intrinsic regulation of bacterial control but rather to differences in the ability of DCs to regulate the differentiation of specific T-cells. Stat3<sup>fl/fl</sup> lym cre APCs release higher levels of IL-6 and IL-23 after stimulation with mycobacterial or other TLR agonists, while secretion of these cytokines is reduced in socs3<sup>fl/fl</sup> lym cre APCs. Stat3<sup>fl/fl</sup> lym cre APCs show improved ability to trigger IL-17 release by mycobacteria-specific T cells while the opposite is observed when using socs3<sup>fl/fl</sup> lym cre APCs. The IL-17 secretion by T cells is also controlled via gp130R signalling, indicating an autocrine or paracrine loop by IL-6 family cytokines. The increased resistance to <i>M. tuberculosis</i> infection of stat3<sup>fl/fl</sup> lym cre mice was IL-17 dependent. Soc3<sup>fl/fl</sup> lym cre (but not stat3<sup>fl/fl</sup> lym cre) DCs improved capacity of mycobacteria-specific T cells priming <i>in vivo</i>.

https://doi.org/10.1371/journal.ppat.1006809.g007

We observed an increased IFN-γ secretion by antigen-specific T cells incubated with STAT3-deficient, mycobacteria-infected APCs. However, IFN-γ responses were not increased <i>in vivo</i>. Whether this is due to the present of cytokines that might stimulate IL-17 responses while antagonizing T<sub>H1</sub> cells (such as for example TGF-β) remains to be explored. Whereas in vitro cultures used provide a proper tool to gain mechanistic insights, the diversity of populations, the tissue localization and the balance between the host immune responses and mycobacteria in the chronic infection might account for differences observed between <i>in vitro</i> responses and the control of infection in mice.

The increased resistance to <i>M. tuberculosis</i> in stat3<sup>fl/fl</sup> lym cre mice is mirrored by data showing that mice with SOCS3 deficiency in myeloid cells display reduced resistance to TB and toxoplasmosis [21, 44]. Here we show that socs3<sup>fl/fl</sup> cd11c cre mice. CD11c cre in these mice has been shown to be expressed in ca 90% of splenic DCs, compared with <10% of lymphocytes and <1% of myeloid cells such as granulocytes [25]. Thus, these animals in which SOCS3 is deleted in DCs but not in inflammatory macrophages and neutrophils [25] are also more susceptible to infection with <i>M. tuberculosis</i>. This supports that the major role of STAT3 and SOCS3 in myeloid cells in the control of infection with <i>M. tuberculosis</i> is not due to an altered ability of SOCS3 or STAT3-deficient macrophages to control the growth of the intracellular mycobacteria <i>in vitro</i>, as shown here and ref [21].

To our knowledge, this is the first report showing that STAT3 deficiency in myeloid cells promotes IL-17 secretion by antigen-specific T cells <i>in vitro</i> and <i>in vivo</i>. Such a role was related to the increased secretion of T<sub>H17</sub> inducing IL-6 and IL-23 by STAT3-deficient APCs. The increased expression of IL-6 and IL-23 in stat3<sup>fl/fl</sup> lym cre APCs was not restricted to the infection with attenuated or virulent mycobacteria since, it was observed after incubating mutant APCs with different TLR agonists or bacterial lysates, confirming previous data [45]. The opposite effect was observed using socs3<sup>fl/fl</sup> lym cre BMM, which were poor inducers of IL-17 secretion by mycobacteria-specific T cells. In relation to this, DC that secrete IL-12p40 (required for T cell differentiation into Th17 or Th1) in the lymph nodes of mycobacteria infected mice are primarily uninfected [46].

Since IL-6 can be produced by various hematopoietic and non-hematopoietic cells, we suggest that APCs are relevant cellular sources of IL-6 for the differentiation of IL-17 secreting cells during infection. Moreover, our data using gp130<sup>−/−</sup> BMDCs indicate that DC-derived IL-6 acts in an autocrine/paracrine manner on DCs to regulate their ability to stimulate IL-17 secretion by T cells. A role for gp130/IL6/STAT3 pathway in susceptibility to <i>M. tuberculosis</i> has been previously determined. The high susceptibility of gp130<sup>−/−</sup> mice to infection with <i>M. tuberculosis</i> was not observed in gp130<sup>−/−</sup>stat3<sup>−/−</sup> or gp130<sup>−/−</sup>stat3<sup>−/−</sup> mice [21].
Our observations on stat3\(^{0/0}\) lym cre mice are reminiscent of those seen in il10\(^{-/-}\) or anti-IL-10R mAb treated mice that resulted in enhanced lung T\(_{11}\) and T\(_{17}\) responses after BCG vaccination [47]. Depletion of IL-10 resulted in elevated protection to \(M\). tuberculosis in some studies but not others [47–50]. However different to our model, IL-10 might not only impair the functions of APCs but is also secreted by T cells and has a direct inhibitory effect on T\(_{11}\) or T\(_{17}\) cells [51].

We showed that the improved \(M\). tuberculosis control in stat3\(^{0/0}\) lym cre is IL-17-mediated, since administration of neutralization anti-IL-17RA antibodies abrogated differences in bacterial burden between mutant and control mice. IL-17 might contribute to long term protection, control of infection after vaccination or control of hypervirulent strains of \(M\). tuberculosis [52–55]. IL-17 has been suggested to induce of protective T\(_{11}\) responses against mycobacterial infection [52, 56]. IL-17 has been also shown to mediate CXCL13 induction in the lung, a chemokine that contributes to the localization of pro-inflammatory cytokine-producing CXCR5+ T cells within lymphoid structures, promoting those macrophage activation and mycobacterial control [53, 57].

However, other studies have indicated that IL-17 is dispensable after primary infection with \(M\). tuberculosis [58]. In line with the later observations, we observed similar \(M\). tuberculosis load in lungs or spleens of WT mice treated or not with anti–IL-17RA.

Levels of MHCII and CD80 and CD86 were lower on socs3\(^{0/0}\) lym cre BMDCs after mycobacterial stimulation confirming previous findings showing reduced MHCII and co-stimulatory molecules after LPS stimulation of SOCS3-deficient BMDCs [59]. Furthermore, the activation of mycobacteria specific p25-tg T cells was also diminished in MLN from \(M\). tuberculosis-infected socs3\(^{0/0}\) lym cre mice as compared to controls. Of importance, p25-tg T cell proliferation was not detectable in the MLN of mice infected with an Ag85b deficient strain of \(M\). tuberculosis indicating the specificity of p25-tg T cell priming [60]. The Ag85b KO \(M\). tuberculosis strain grew in the lungs and disseminated to the MLN at a rate equivalent to that of wild-type bacteria. Instead, stat3\(^{0/0}\) lym cre and control BMDCs expressed similar levels of MHC-II and co-stimulatory molecules after mycobacterial stimulation in vitro and similar levels of activated antigen-specific T cells in vivo. Different to these results, STAT3 deficient APCs have been shown increased MHCII levels after IL-6 stimulation [61].

Finally, the role of STAT3 in T cells in regulation of antigen-specific IFN-\(\gamma\) and IL-17 T cell responses was investigated. Contrary to the role of STAT3 in APCs, IL-17 secretion was hampered in mycobacteria-specific STAT3-deficient T cells. STAT3 is required for the responses to both IL-6, IL-21 and IL-23 and for the expression of ROR\(\gamma\) by T cells [62]. Instead, SOCS3-deficient antigen-specific T cells secreted higher IL-17 levels as previously reported in other systems [35], while IFN-\(\gamma\) responses were inhibited. Thus, while the role of STAT3 in T cells in the control of \(M\). tuberculosis remains to be studied, these results illustrate the pleiotropic effect of STAT3 in regulation of infection-induced immune responses in different cell types.

In summary, we here showed using SOCS3- and STAT3-deficient mice that STAT3 in myeloid cells is detrimental for the control of infection with \(M\). tuberculosis. Surprisingly, this occurs via impairing secretion of IL-17 by antigen-specific T cells (Fig 7E).

**Materials and methods**

**Ethics statement**

The animals were housed and handled at the Dept. of Microbiology, Tumor and Cell Biology and the Astrid Fagreus Laboratory, Karolinska Institute, Stockholm, according to directives and guidelines of the Swedish Board of Agriculture, the Swedish Animal Protection Agency,
and the Karolinska Institute (djurskyddslisten 1988:534; djurskyddsförordningen 1988:539; djurskyddsmyndigheten DFS 2004:4). The study was performed under approval of the Stockholm North Ethical Committee on Animal Experiments permit number N397/13 and N487/11. Animals were housed under specific pathogen-free conditions.

**Mice**

Mice containing loxP-flanked *stat3* and *socs3* alleles have been described before [43]. For a myeloid-specific deletion these were bred with transgenic *lysm* cre mice [63]. *Socs3*fl/fl mice were also bred with *cd11c cre* transgenic animals. *Stat3*fl/fl or *socs3*fl/fl littermates negative for cre expression were used as controls for all experiments. *Gp130*F/F mice with a homozygous substitution of tyrosine (Y)757 to phenylalanine (F) within the common IL-6 family receptor gp130 abrogating the SOCS3 binding site have been described before [64]. Transgenic T cell receptor *p25-tg* mice with a T-cell receptor specific for peptide 25 (aa 240–254) of mycobacterial Ag85B on H2b haplotype were used [65]. *p25-tg rag2−/−* mice expressing ECFP were generated by crossing *p25-tg* with *rag1−/−* mice [65] with ECFP mice on a *rag2−/−* background (kindly provided by Dr. Ronald Germain, NIAID, NIH). The ECFP expression co-localized with Vβ11 used by p25tg T cells [65]. *Socs3*fl/fl *lck cre* and *stat3*fl/fl *lck cre* mice deficient in SOCS3 and STAT3 in T cells were crossed with *p25-tg* mice to generate *p25-tg socs3*fl/fl *lck cre* and *p25-tg stat3*fl/fl *lck cre* mice. *p25-tg lck cre* mice were also obtained and used as controls.

**Infection and infectivity assay**

BCG Montreal and *M. tuberculosis* Harlingen were grown in Middlebrook 7H9 (Difco, Detroit, MI) supplemented with albumin, dextrose, catalase and, for BCG cultures, 50 μg/ml hygromycin (Sigma, St. Louis, MO). Mice were infected with 250 *M. tuberculosis* Harlingen strain by aerosol using a nose-only exposure unit (In-tox Products, Uppsala, Sweden)[66]. Bacteria were quantified on Middlebrook 7H11 agar containing 10% enrichment of oleic acid, albumin, dextrose, catalase, 5 μg of amphotericin B per ml and 8 μg/ml polymyxin B grown for 3 weeks at 37°C.

**Generation of mouse bone marrow-derived macrophages**

Bone marrow was extracted from tibia and femurs of mice and resuspended in DMEM containing glucose and supplemented with 10% FCS and 30% L929 cell-conditioned medium (as a source of macrophage-colony stimulating factor). Bone marrow cells were passed through a 70 μm cell strainer, plated and incubated for 6 days at 37°C, 5% CO₂. Bone marrow-derived macrophage (BMM) cultures were then washed vigorously to remove non-adherent cells, trypsinized, counted and cultured for one day at 37°C in 24, 12 or 6 well plates. We have previously shown that these BMM are F4/80+, CD14+ and Mac-3+ [67].

**Quantification of intracellular mycobacteria**

In order to quantify intracellular *M. tuberculosis* uptake and growth, BMM cells were plated on glass slides at 2.10⁵ cells per well in 24 well plates, incubated for 4 h with *M. tuberculosis* (MOI 2) and washed with PBS for 3 times to remove the extracellular bacteria before either fixation or replacing the medium. Three days after infection cells were washed with PBS, fixed with 2% PFA and stained with phalloidin to label F-actin (Life technologies, 1:100), DAPI (1:500) and auramine-rhodamine T to label mycobacteria (BD). Micrographs from infected macrophages (400X) were obtained and a total of at least 1000 BMM from 3 independent cultures and categorized as infected or uninfected. The intracellular *M. tuberculosis* were enumerated. BMM
harboring 5 or more bacteria were considered as containing 5. In some cultures, mycobacterial CFU from BMM 6 days after infection were determined.

**Generation of mouse bone marrow-derived dendritic cells**

Mouse bone marrow-derived dendritic cells (BMDC) were differentiated as previously described [68]. Briefly, bone marrow was extracted from tibia and femurs and cell suspensions cultured in RPMI-1640 medium containing 10% FCS and 2 ng/ml GM-CSF (Peprotech, Rocky Hill, NJ). Fresh medium and cytokine were replaced after 3 days. After six days of culture, loosely adherent cells were harvested and seeded in concentrations for infection. Harvested cells were further selected for CD11c expression with magnetic beads (Miltenyi Biotech) before seeding.

**T cell priming in vitro**

BMDC or BMM were stimulated with either live or heat killed BCG, *M. tuberculosis* or Ag85b peptide in presence of LPS for 6 h. Then, cells were washed and co-incubated with p25-tg CD4+ lymph node transgenic T cells from *rag2*−/− p25-Tg mice (at a ratio of 4:1 BMDC). The cultures were further incubated for 24–48 hs at 37°C 5% CO₂. At these time points the concentration of IFN-γ and IL-17 in the supernatants was measured by ELISA.

**Real time PCR**

Transcripts were quantified by real time PCR as previously described[66]. *Hprt* was used as a control gene to calculate the ΔCt values for individual samples. The relative amount of cytokine/ hprt transcripts was calculated using the 2^(-ΔΔCt) method. These values were then used to calculate the relative expression of cytokine mRNA in uninfected and infected cells and tissues.

**Flow cytometry and intracellular cytokine staining**

Lungs were perfused with PBS through the heart before removal from mice. Lungs were mechanically minced into small pieces and digested with 3 mg/ml Collagenase D and 30 µg/ml DNase I for 1 h at 37°C, and single-cell suspensions prepared by filtering lung tissue through 70-µm nylon cell strainers. To further remove impurities cells were loaded in 40/ 70% Percoll gradient in PBS and centrifuged 30 min room temperature. The cells at the interphase were collected and washed. Single spleen cell suspensions were obtained by mechanical disruption, lysis of erythrocytes and straining over a 70-µm nylon mesh. Lung, lymph node and spleen cells were stained for CD3, CD4, CD8, γδ- TCR, CD62L, CD69, CD44, CD11b, CD11c, Ly6C and Ly6G (all eBioscience) and fixed before acquisition.

For determination of IFN-γ and IL-17-producing cells, lung cells were incubated with PPD or with 50 ng/ml phorbol myristate acetate (PMA) and 2 µg/ml ionomycin (Sigma) for 6 or 18 h at 37°C. Brefeldin (10 µg/ml) was added to the cultures the last 4 h of stimulation. Cells were then stained with cell population-specific antibodies, and live/ dead staining, fixed, permeabilized using leukocyte permeabilization reagent IntraPrep™ (Immunotech, Marseille, France) and further stained with anti-IL-17a or anti-IFN-γ (eBioscience). Data were acquired in a CyAn™ ADP (Beckman Coulter) or an LSRII Flow cytometry and analyzed with FlowJo software (Tree star Inc., Ashland, OR).
Histopathological analysis

Formalin fixed left lungs of mice experimentally inoculated with *M. tuberculosis* were blocked on paraffin. From each lung sample 4 sections were obtained, one longitudinal along the long axis of the lobe and 3 across/transversal of the remaining piece of lung.

The blocks were processed and sections were stained with haematoxylin-eosin. All sections were interpreted by the same pathologist (D. G-W.) and scored semi-quantitatively, blinded to the variables of the experiment.

The following features were scored:

- Lung area occupied with granulomas (% of the total area of the section)
- Lung area free of lesions or area of healthy lung (% of the total area of the section)

Statistics

The Mann Whitney test for the bacterial CFU load in vivo and of the ICS analysis. For each experiment, 8–10 control and 8–10 mutant mice were infected. We performed separated experiments for 4 and 8 weeks post infection. One of two independent experiments showing similar results is shown.

The analysis of cytokine secretion or mRNA, histopathological scores and frequencies was done using the Student’s t test for unpaired samples. All *in vitro* experiments were performed at least twice. A two-way ANOVA was used to compare the differences in IL-17 secretion between genotypes, as well as between cells that co-secrete IFN-γ or not.

Supporting information

S1 Fig. Frequency and numbers of pulmonary neutrophils in *stat3β/β lysm cre* and control mice at 14 weeks after infection with *M. tuberculosis*. The frequency (A) and numbers (B) of CD11b+CD11cLy6CdimLy6G+ neutrophils in lungs *stat3β/β lysm cre* and *stat3β/β* mice at 14 weeks after infection with *M. tuberculosis ± SEM* are shown (n = 4 mice per group); representative dot plots of neutrophil staining in lungs are shown (C).

(TIFF)

S2 Fig. Frequency of PMA/ionomycin-stimulated IFN-γ-secreting CD4+ and CD8+ T cells from lungs of *stat3β/β lysm cre* and *stat3β/β* mice 4 and 8 weeks after infection with *M. tuberculosis*. The mean frequency of PMA/ ionomycin-stimulated IFN-γ secreting CD4+ and CD8+ lung T cells from *stat3β/β lysm cre* and *stat3β/β* mice at 4 (B) and 8 (C) weeks after infection with *M. tuberculosis ± SEM* was measured by FACS (n = 4 per group).

(TIFF)

S3 Fig. Frequency of pulmonary lymphoid cell populations in *stat3fl/fl lysm cre* and control mice after infection with *M. tuberculosis*. The mean frequency of CD4+, CD8+ and γδ+ cells within lung CD3+ T cells from *stat3β/β lysm cre* and *stat3β/β* mice 8 weeks after infection with *M. tuberculosis was measured by FACS (n = 4 per group) (A).

The mean frequency of PPD and PMA/ ionomycin-stimulated IL-17 secreting γδ+ pulmonary T cells from *stat3β/β lysm cre* and *stat3β/β* mice at 4 (B) and 8 (C) weeks after infection with *M. tuberculosis was measured by FACS (n = 4 per group).

(TIFF)

S4 Fig. *Il17* and *il22* mRNA accumulation in lungs from *stat3β/β lysm cre* and control mice before and 14 weeks after infection with *M. tuberculosis*. The mean fold increase of *il17a* (A), *il22* (B) mRNA ± SEM was measured by real time PCR in the total RNA from lungs of *stat3β/β* mice.
lysm cre and stat3β/β mice at 14 weeks after M. tuberculosis infection (n = 5 per group *p<0.05 Student’s t test).

(TIFF)

S5 Fig. Levels of il6 and il23p19 mRNA in stat3β/β lysz cre and stat3β/β after stimulation with different TLR agonists. The mean fold increase of il6 (A) and il23p19 (B) ± SEM were measured by real-time PCR in triplicate cultures of stat3β/β lysz cre and stat3β/β BMDCs 6 h after stimulation with either LPS, CpG or Pam3K (* p<0.05 and ***p<0.001 Student t test).

(TIFF)

Acknowledgments

We thank suggestions and comments to our study from Dr Benedict Chambers and Dr Susanne Nylen. We acknowledge the excellent technical help from Berit Olsson, Helen Braxenholm, Torun Söderberg and Ida Fahlen. We thank Dr Antonio Rothfuchs for suggestions and facilitating access to p25-tg mice. The rat anti-mouse IL-17RA (M751) blocking Ab was kindly provided by Amgen.

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