Title

SOCS3 overexpression in T cells ameliorates chronic airway obstruction in a murine heterotopic tracheal transplantation model

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A brief, specific, and informative title

The impact of SOCS3 overexpression in airway obstruction

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Abstract

Purpose: Suppressor of cytokine signaling-3 (SOCS3) is a negative feedback inhibitor of cytokine signaling with T cell-mediated immunosuppressive effects on obliterative bronchiolitis (OB). In this study, we aimed to investigate the impact of T-cell specific overexpression of SOCS3 using a murine heterotopic tracheal transplantation (HTT) model.

Methods: Tracheal allografts from BALB/c mice were subcutaneously transplanted into wild-type C57BL/6J (B6; WT) mice and SOCS3 transgenic B6 (SOCS3TG) mice. Tracheal allografts were analyzed by immunohistochemistry and quantitative polymerase chain reaction assays at day 7 and 21.

Results: At day 21, allografts in SOCS3TG mice showed significant amelioration of airway obstruction and epithelial loss compared with allografts in WT mice. Intragraft expression of

IFN-γ and CXCL10 was suppressed and IL-4 was enhanced in SOCS3TG mice at day 7. T-bet levels was lower in SOCS3TG allografts compared to WT allografts at day.

Conclusion: We revealed that overexpression of SOCS3 in T cells effectively ameliorates OB development in a murine HTT model by inhibiting the Th1 phenotype in the early phase. Our results suggest that the regulation of the T cell response, through the modulation of SOCS expression, has potential as a new therapeutic strategy for chronic lung allograft dysfunction (CLAD).

Abbreviations

CLAD, chronic lung allograft dysfunction; CXCL, C-X-C motif chemokine ligand; IL, interleukin; IFN, interferon; JAK/STAT, Janus kinase-signal transducer and activator of transcription; OB, obliterative bronchiolitis; RAG1, recombination activating 1; SEM, standard error of the mean SOCS, Suppressor of cytokine signaling; Th, T helper cell; Treg,

regulatory T cell

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Introduction

Despite clinical advances, the outcome of lung transplantation is worse than that of other solid organ transplantations. Chronic lung allograft dysfunction (CLAD) is the major cause of death after lung transplantation: it is developed in 50% of recipients within 5 years from transplant, limiting long-term survival [1]. CLAD is commonly presented as bronchiolitis obliterans syndrome (BOS), defined by sustained decline in the forced expiratory volume in one second (FEV1.0). Histologically, BOS is manifested by obliterative bronchiolitis (OB), characterized by lymphocyte infiltration and fibroproliferation, resulting in obstruction of the small airways and indicating chronic rejection [2] [3]. Unfortunately, no effective prevention and treatment of OB have been established yet.

Recently, an increasing number of studies have focused on the role of specific cytokine and lymphocyte subsets in rejection, to elucidate the mechanism of alloimmune response. Among

numerous cytokines, the importance of Th1 cytokines has been recognized because of its increase in patients with BOS [4] [5] [6] [7]. However, it has been reported that rejection occurs also under depletion of Th1 [8] or IFN-γ [9], the major Th1 cytokine, suggesting that the secretion of a single cytokine by multiple sources and the overlapping function between different cytokines are barriers to the effective treatment.

The SOCS family of proteins function as negative regulators of intracellular signaling [10] [11] and have a critical role in T cell differentiation, maturation, and function via the regulation of the JAK-STAT pathway [12]. Among the eight members of the SOCS protein family, we focused on SOCS3, which has an immunosuppressive function in adaptive immunity via regulation of T cell subsets, such as Th1 and Th2 [11]. Overexpression of SOCS3 skews the Th1/Th2 balance towards the Th2 phenotype, resulting in inhibition of the Th1 response [13]. Additionally, SOCS3 exerts an inhibitory function on Th17 [14] [15] [16]

[17], whose role in rejection after lung transplant has been recently elucidated [18] [19].

A heterotopic tracheal transplant (HTT) model was first developed in 1993 for the investigation of OB [20]. In this model, tracheal allografts undergo a process similar to OB after lung transplant, showing identical pathological features: initiation with ischemia-induced loss of the respiratory epithelium, infiltration of inflammatory cells into the grafts [21, 22] followed by fibroproliferative change involving smooth muscle cells and fibroblasts, resulting in occlusion of the intratracheal area [23]. Because of its simplicity and reproducibility, this model has been widely used to uncover the mechanisms of OB [23] and analyze the therapeutic effect of drugs [24] [25] [26] [27] [28], contributing to rapid progress in research on OB.

In this study, we hypothesized that the immunosuppressive effects of SOCS3 overexpression through T cell regulation attenuate OB, thus maintaining a balance between specificity and

effectiveness, and elucidated the impact of SOCS3 overexpression using the murine HTT model.

Methods

1. Mice

The SOCS3 transgenic (SOCS3TG) mice have been previously described [29, 30]: they bear the myc-tagged *SOCS3* transgene under the control of the lck proximal promoter and the human immunoglobulin heavy chain (Eμ) enhancer [13, 31]. Transgenic mice were backcrossed with C57BL/6J mice. Recombination activating gene 1 (RAG1) knockout mice (on a C57BL/6J background) were a gift from Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan). BALB/c mice were from CLEA Japan (Tokyo, Japan). C57BL/6J mice, purchased from CLEA Japan and bred in our facilities, were used as wild-type mice (WT).

All mice were transferred to the Advanced Science Center Department of Animal Resources, Okayama University (Japan) and bred in a specific pathogen free environment. Eight-to-sixteen weeks old mice were used for the experiments. The animal protocols were approved by the Animal Committee of Advanced Science Center, Department of Animal Resources, Okayama University.

2. Subcutaneous HTT

HTT was performed as previously described [32] [33] [34]. In brief, tracheal grafts, from the cricoid cartilage to bifurcation, were recovered from CO_2-euthanized donor mice, rinsed and placed in PBS on ice until implantation. Recipient mice were anesthetized using isoflurane. Tracheal grafts were transplanted into a subcutaneous pocket, bluntly created on the dorsal upper site of recipient mice. The wound was closed with sutures. Recipient mice were

maintained without immunosuppression until the analysis. Five or more mice were used in each transplant group for every experiment.

3. Histology

Grafts were collected after euthanasia of recipient mice using CO_2, fixed in 10% formalin, and embedded in paraffin. Thin sections were stained with hematoxylin-eosin and Masson's trichrome staining. The pathological score, was decided by a pathologist in blinded fashion and was composed of three elements, as follows. (1) Epithelial damage: 0, no change; 1, regenerative change; 2, < 50% loss; 3, 55-99% loss; 4: 100% loss. (2) Inflammatory cell infiltration into lamina propria: 0, < 10 cells/hpf; 1, 10-19 cells/hpf; 2, 20-50 cells/hpf; 3, > 50 cells/hpf. (3) Lamina propria changes: 0, no thickening; 1, edema without spindle cell proliferation; 2, spindle cell proliferation in < 50% area; 3, spindle cell proliferation in ≥ 50%

area.

4. Immunohistochemistry

Formalin-fixed and paraffin-embedded thin sections were deparaffinized. Endogenous peroxidase activity was blocked using methanol/hydrogen peroxide. After antigen retrieval with citric acid or proteinase K, immunohistochemistry was performed using the following primary antibodies: rat anti-human CD3 (Bio-Rad Laboratories, Hercules, CA, USA) and rat anti-mouse B220 (BD Biosciences, San Jose, CA, USA). To detect the immunocomplexes, the Polink-2 Plus HRP Detection Kit (GBI Labs, WA, USA) was used, followed by visualization with the Liquid DAB + Substrate Chromogen System (DAKO, Carpinteria, CA, USA) and nuclear staining with hematoxylin.

5. RNA isolation and quantitative polymerase chain reaction (qPCR)

Trachea grafts were collected and stored in RNAlater (Qiagen, Hilden, Germany). mRNA was extracted with TRIsure™ (Nippon Genetics, Tokyo, Japan) according to the manufacturer's instructions. After measuring mRNA concentration, reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). For qPCR, Taqman™ gene expression assays (Applied Biosystems) were used to quantify the mRNA level of β actin (Mm00607939_s1), IFN-γ (Mm01168134_m1), C-X-C motif chemokine ligand 10 (CXCL10; Mm00445275_m1), IL-4 (Mm00445259_m1), IL-13 (Mm00434204_m1), using StepOnePlus Real-Time PCR system (Applied Biosystems). For measuring the expression of transcription factors, PrimeTime qPCR Assays (Integrated DNA Technologies, Illinois, USA) specific for β actin (NM_007393) and T-bet (NM_019507) were used. The Ct value of the target gene was

normalized to that of β actin and the relative expression level was calculated setting the value of isografts at day 7 as 1.

6. Luminal obstruction ratio

Hematoxylin-eosin stained graft sections were photographed at ×40, ×100, ×200, or ×400 magnification using an OLYMPUS BX43 microscope (OLYMPUS, Tokyo, Japan). The intra-cartilage area and free lumen were measured in sections from the center part of the tracheal graft using CellSens (OLYMPUS). Luminal obstruction ratio was calculated as follows: (1 - free lumen / intra-cartilage area) * 100.

7. Statistics

The data were analyzed with the GraphPad Prism 6 software (GraphPad Software, San Diego,

CA, USA) using two-tailed Student's t test for the comparison of two groups. Allografts in WT mice were compared to isografts. Allografts in SOCS3TG mice were compared to allografts in WT mice. The data are reported as the mean ± SEM. $P < 0.05$ was considered significant.

Results

1. Allografts developed OB at day 21

First, we determined the conditions to investigate OB in the heterotopically transplanted tracheal allografts in our condition. Tracheal grafts from C57BL/6J (for isotransplant) or Balb/c (for allotransplant) mice were subcutaneously transplanted into B6 recipients and histologically evaluated at day 7 and 21. Allografts in rag1-/-mice, which do not develop OB because of lacking whole lymphocytes, were used as a negative control for OB. Luminal

obstruction due to fibroproliferation and epithelial loss were observed in allografts at day 21 (Fig. 1a), whereas no obvious change was observed in isografts. Grafts were quantitatively assessed by determining the obstruction ratio and the pathological scores (epithelial damage, inflammatory cell infiltration and lamina propria changes scores, as described in the Materials and Methods section). At day 21, allografts in WT mice showed higher obstruction ratio and epithelial damage score compared with both isografts and allografts in rag1-/- mice (Fig. 1b). However, the inflammatory cell infiltration and lamina propria changes scores in allografts in WT mice were not significantly higher than those in both negative controls. These results demonstrated the development OB in allografts at day 21 and the validity of the obstruction ratio and epithelial damage score for graft evaluation. Next, we investigated the effect of the T cell-specific overexpression of SOCS proteins in OB development in comparison to the WT (Fig. 1). At day 21, allografts transplanted into SOCS3TG mice

showed attenuated luminal obstruction and preserved epithelia, as indicated by the lower obstruction ratio and epithelial damage score, compared with allografts transplanted in WT mice.

2. Changes in Th1 and Th2 cytokine expression in allografts in SOCS3TG mice at day 7

We hypothesized that the inhibition of OB in SOCS3TG mice was the result of Th1 inhibition in the early inflammatory phase. To test this hypothesis, we measured the expression of representative Th1 and Th2 cytokines at day 7 (Fig. 2a). and 21 (Fig. 2b) after transplant. The relative expression of IFN-γ and CXCL10 (a Th1 chemokine also known as interferon-γ-inducible protein 10) was increased in WT allografts compared to isografts at day 7 (IFN-γ: 290 ± 103 vs. 1.1 ± 0.22, $p = 0.023$; CXCL10: 67 ± 16 vs. 1.3 ± 0.55, $p = 0.0041$) and day 21 (IFN-γ: 234 ± 89 vs. 13.2 ± 11, $p = 0.039$; CXCL10: 23 ± 5.4 vs. 5.8 ± 3.8, $p = 0.028$). In SOCS3TG allografts, the levels of both Th1 cytokines were significantly lower than those in

WT allografts at day 7 (IFN-γ: 30 ± 28, p = 0.040; CXCL10: 16 ± 14, p = 0.046, respectively). Additionally, the expression of IL-4, a Th2 cytokine, was comparable in isografts and WT allografts and higher in SOCS3TG allografts at day 7 (SOCS3TG vs. WT, 9.4 ± 4.0 vs. 1.5± 0.28, p = 0.041). At day 21, IFN-γ and CXCL10 levels in SOCS3TG allografts were similar to those in WT allografts. Interestingly, IL-13, another Th2 cytokine, also elevated in SOCS3TG allografts not at day 7 but at day 21 (SOCS3TG vs. WT, 23.7 ± 3.93 vs. 5.6 ± 3.53, p = 0.0083) indicating its role in fibrosis which occurs in late phase.

3. The expression of T-bet is lower in SOCS3TG allografts at day 7

To examine the change of T cell subset in SOCS3TG allografts during inflammation, the grafts at day 7 were further evaluated. Allografts in SOCS3TG mice exhibited inflammatory cell infiltration into the lamina propria to the same extent as the WT allografts, as confirmed by the pathological score (2.6 ± 0.24 vs. 2.0 ± 0.45, p = 0.27, data not shown).

Immunohistochemistry revealed predominant infiltration of CD3-positive T cells into the WT allografts, while very few B cells (B-220-positive) were found in allografts of both groups, demonstrating that the predominant effector cells in early inflammation are T cells(Fig. 3a). In SOCS3TG allografts, while the number of CD3-positive cells was unchanged (Fig. 3b), the expression of T-bet, a Th1 transcription factor, was lower than that in WT allografts (Fig. 3c, 0.59 ±0.094 vs. 7.3 ± 1.8, $p = 0.0052$) indicating a decline in the Th1 population

Discussion

BOS is one of the critical complications in lung transplant recipients and its histological manifestation is known as OB. It has been recognized the underlying mechanism of OB is allogenic rejection where T cells, especially Th1 cells, function predominantly. Here we present overexpression of SOCS3 in recipient T cell attenuated the development of OB and

found this inhibitory effect occurred following the inhibition of Th1 response in early phase using HTT model.

The number of reports investigating the role of specific T cell subset and cytokine has been increasing to solve the issue of immunosuppression, such as severe infection and malignancy, by targeting particular T cell subset or cytokine. The present study highlighted the role of two T cell subsets in OB; Th1 and Th2.

Our result showed that the inhibitory effect of SOCS3 overexpression in T cell on OB was derived from inhibition of Th1. However, previous reports of depleting Th1 or Th1 cytokine showed no effect on rejection inconsistent with our findings. OB was developed in IFN-γ KO mice and lung allograft showed severe acute rejection in T-bet-deficient mice, which have impaired Th1 response because of the lack of T-bet, a Th1 transcriptional factor [8][29]. These facts suggest the complexity of T cell subsets and cytokines resulting in compensation

of the lack of single subset and cytokine. The reason of the attenuation of OB in SOCS3TG mice might be the more function of SOCS3 than inhibiting single cytokine or subset. For example, SOCS3 has been shown to inhibit IL-17 responses as well [14] [15] [16] [17], which is recently considered an important factor in rejection [19] [18] [35]; this might contribute to the inhibitory effect of SOCS3 overexpression on OB. Based on our results and these facts, SOCS3 has a potential as an effective therapeutic target of OB.

In terms of the role of Th2, the importance of the Th1/Th2 switch from early inflammation to fibroproliferation has been shown in research on other fibrotic diseases, which also cause OB [36] . In the OB after lung transplant, although the evidence showing the involvement of Th2 cytokines has been accumulating [37] [38], the role of Th2 cells remains unclear. Our results showed OB attenuation in SOCS3TG allografts, together with the enhancement of the Th2 response as indicated by elevated levels of IL-4 and IL-13. This suggests that

suppression of the Th1 is crucial for inhibition of OB counteracting the Th2 enhancement.

This is the first report focusing on the role of SOCS3 in lung transplant. Very few reports have been found relating the impact of SOCS3 in transplantation: one demonstrated that SOCS3 deficiency promotes graft-versus-host disease after hematopoietic stem cell transplantation [39] and two showed that the induction of SOCS3 delays rejection after islet [40] or cardiac transplant [41]. Consistent with those reports, our findings supported the inhibitory effects of SOCS3 on post-transplant rejection.

We acknowledge that the use of the HTT model in this study has some limitations such as the inability to validate the effect in the whole lung in vivo because of the absence of air flow and circulation responsible for blood gas exchange, and limited analysis because of the very small size of tracheal graft. However, HTT model has great advantages of the high reproducibility derived from the unnecessity of special equipment and difficult techniques,

that are needed in orthotopic lung transplant model, and the capability of physiological observation to figure out the phenotype of airway obstruction occurs in chronic phase. This model was chosen in this study based on our purpose of initial investigation of the impact of the recipient T cell alteration on development of OB. However, the detailed mechanism of the inhibitory effect of SOCS3 overexpression has not been elucidated in this study. For further investigation, different models such as the orthotopic transplant model would be beneficial.

In conclusion, overexpression of SOCS3 ameliorated the airway obstruction in the murine HTT model. Our results suggest that the regulation of T cell response, through the modulation of SOCS3 expression, would have a therapeutic potential for CLAD after lung transplantation.

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Figure Legends

Figure 1. SOCS3 overexpression in T cells attenuates OB at day 21 after heterotopic tracheal transplant. (a) Representative images of tracheal grafts at day 21 after transplantation. Sections were stained with hematoxylin and eosin (HE, top row) or Masson's Trichrome (MT, middle and bottom row). Scale bar, 300 μm. Asterisk, luminal obstruction with fibroproliferative tissue. Arrow, epithelial loss. (b) Quantitative assessment of graft histology using the obstruction ratio and the epithelial damage score demonstrated attenuation of OB in SOCS3TG recipients compared with WT recipients. Scale bar, 100 μm; n = 5-6 per group.

Figure 2. SOCS3TG allografts show suppressed Th1 and enhanced Th2 cytokine expression at day 7. (a) Quantitative PCR revealed lower expression of IFN-γ and CXCL10 and higher levels of IL-4 in SOCS3TG allografts compared to WT at day 7. n ≥ 5 per group. (b) Quantitative PCR as in (a). The effect of SOCS3 overexpression on of IFN-γ, CXCL10 and

IL-4 expression disappeared at day 21. IL-13 level was elevated in SOCS3TG allografts. n = 5 per group.

Figure 3. In SOCS3TG allografts at day 7, the number of CD3-positive cells is unchanged and the expression of T-bet decreases. (a) Representative images of allografts in WT and SOCS3TG recipients at day 7. Immunohistochemistry showed that most of the inflammatory cells infiltrated into the lamina propria were T cells (CD3-positive, middle row) and not B cells (B220-positive, bottom row). Scale bar, 300 μm. (b) The number of CD3-positive T cells infiltrating into the intra-cartilage area at day 7 was counted in images taken at x 200 magnification. The mean of four separate areas was used for each graft. n = 5 per group. (c) Quantitative PCR revealed lower expression of T-bet in allografts in SOCS3TG recipients compared with WT ones. n = 5 per group.

Figure 1

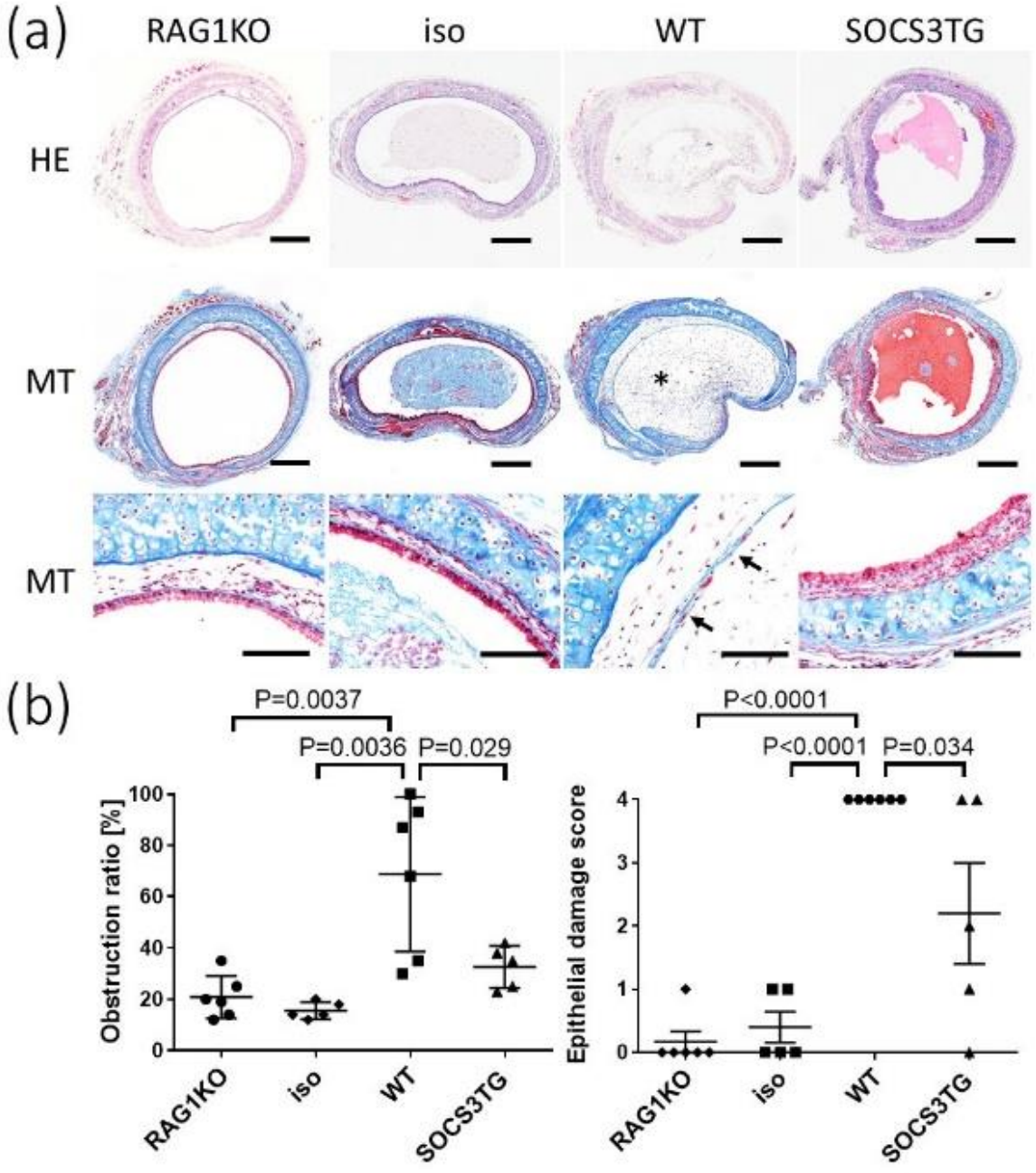

(a)
RAG1KO
iso
WT
SOCS3TG
HE
MT
MT
*
(b)
P=0.0037
P=0.0036
P=0.029
Obstruction ratio [%]
100
80
60
40
20
0
RAG1KO
iso
WT
SOCS3TG
P<0.0001
P<0.0001
P=0.034
Epithelial damage score
4
3
2
1
0
RAG1KO
iso
WT
SOCS3TG

Figure 2

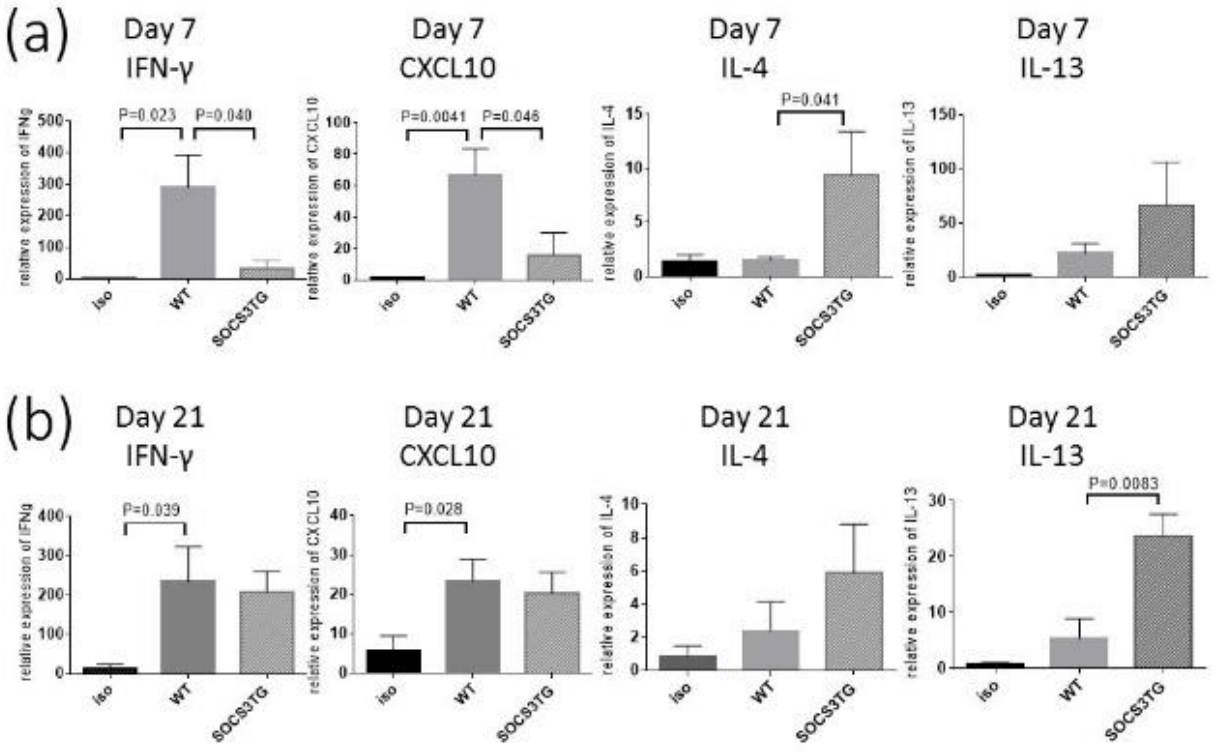
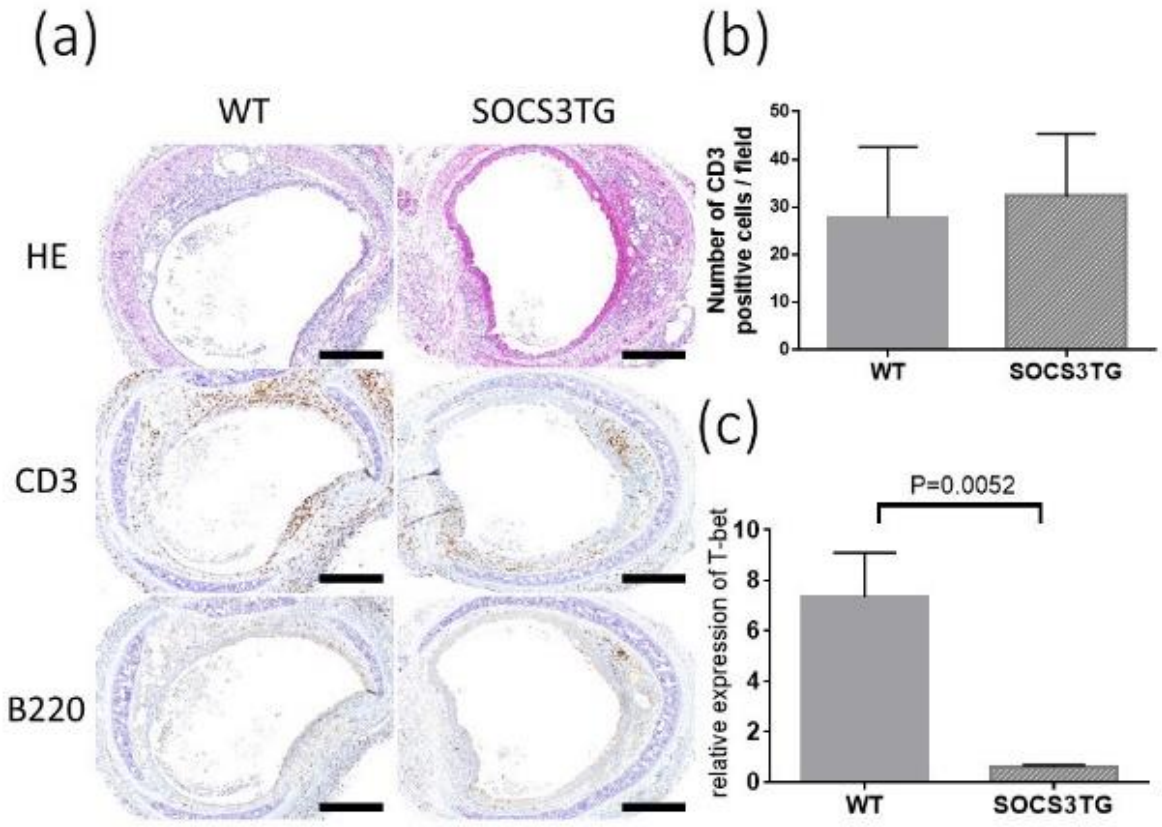

(a)
Day 7
IFN-γ
P=0.023
P=0.040
relative expression of IFNg
iso
WT
SOCS3TG
Day 7
CXCL10
P=0.0041
P=0.046
relative expression of CXCL10
Day 7
IL-4
P=0.041
relative expression of IL-4
Day 7
IL-13
relative expression of IL-13
(b)
Day 21
IFN-γ
P=0.039
Day 21
CXCL10
P=0.028
Day 21
IL-4
Day 21
IL-13
P=0.0083

Figure 3


(a)
WT
SOCS3TG
HE
CD3
B220
(b)
Number of CD3 positive cells / field
50
40
30
20
10
0
WT
SOCS3TG
(c)
P=0.0052
relative expression of T-bet
10
8
6
4
2
0
WT
SOCS3TG