

## LACK OF ST2 ENHANCES HIGH-FAT DIET-INDUCED VISCERAL ADIPOSITY AND INFLAMMATION IN BALB/c MICE

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## DELECIJA GENA ZA ST2 PROMOVIŠE GOJAZNOST I INFLAMACIJU U VISCERALNOM ADIPOZNOM TKIVU BALB/C MIŠEVA NA DIJETI SA VISOKIM SADRŽAJEM MASTI

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### ABSTRACT

*Obesity and obesity-related disorders are strongly associated with a chronic low-grade inflammation that originates from growing visceral adipose tissue during nutrient excess. Although interleukin (IL)-33 may play a protective role in obesity and atherosclerosis, the impact of the IL-33/ST2 axis on metabolic disorders needs to be further elucidated.*

*In this study, we investigated the role of the IL-33/ST2 pathway in high-fat diet (HFD)-induced obesity using ST2-deficient (ST2<sup>-/-</sup>) and wild type BALB/c mice.*

*The deletion of ST2 enhanced systemic and visceral adipose tissue (VAT) inflammation; ST2<sup>-/-</sup> mice that were fed a HFD for 18 weeks had experienced a significantly increased weight gain and had a higher amount of total VAT. More classically activated M1 macrophages and markedly fewer alternatively activated M2 macrophages were observed in the VAT of the HFD-fed ST2<sup>-/-</sup> mice. Additionally, the VAT of the HFD-fed ST2<sup>-/-</sup> mice had an increased percentage of CD3<sup>+</sup> T cells but fewer CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T regulatory cells when compared to the VAT of the low-fat diet-fed controls. The numbers of CD3<sup>+</sup>IL-17<sup>+</sup> and IL-5 positive VAT-derived mononuclear cells were significantly decreased in the HFD-fed ST2<sup>-/-</sup> mice. Serum levels of the proinflammatory cytokines IL-1 $\beta$  and IFN- $\gamma$  were increased in the HFD-fed ST2<sup>-/-</sup> mice, while the levels of IL-6 and CRP did not differ among the groups. Importantly, the levels of the anti-inflammatory cytokines IL-10 and IL-13 were significantly lower in the sera of the ST2<sup>-/-</sup> mice than the levels in the sera of the wild-type controls.*

*Our findings suggest a protective role of IL33/ST2 signaling in high-fat diet-induced adipose tissue inflammation. ST2 deficiency related to nutrient excess is associated with the polarisation of macrophages toward the M1 phenotype and the induction of a Th1-mediated immune response.*

**Key words:** obesity, adipose tissue, inflammation, cytokines, macrophages

### SAŽETAK

*U osnovi patogeneze gojaznosti i metaboličkih poremećaja povezanih sa gojaznošću je hronična sistemska inflamacija niskog stepena koja nastaje u visceralnom adipoznom tkivu (VAT) u uslovima povećanog unosa nutrijenata. Iako rezultati dosadašnjih istraživanja ukazuju na moguću protektivnu ulogu IL-33 u nastanku gojaznosti i ateroskleroze, uloga IL-33/ST2 signalnog puta u patogenezi ovih bolesti je nedovoljno razjašnjena.*

*U ovom istraživanju ispitivali smo ulogu IL-33/ST2 signalnog puta u mišjem modelu gojaznosti indukovane primenom dijetete sa visokim sadržajem masti u ST2 deficijentnih i miševima divljeg soja BALB/c.*

*Delecija gena za ST2 promovira sistemska inflamaciju i inflamaciju u VAT-u što se ogleda u porastu telesne mase i uvećanju količine VAT-a tokom 18 nedelja primene dijetete sa visokim sadržajem masti. Proinflamatorni milje u VAT-u ST2<sup>-/-</sup> miševa na ishrani bogatoj mastima karakteriše povećana zastupljenost klasično aktiviranih M1 makrofaga, uz smanjeno prisustvo alternativno aktiviranih M2 makrofaga. Pored toga, dijeteta sa visokim sadržajem masti značajno je uticala na povećanje zastupljenosti CD3<sup>+</sup> T limfocita, dok je prisustvo CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatornih T limfocita bilo značajno sniženo u VAT-u ST2<sup>-/-</sup> miševa u odnosu na ST2<sup>-/-</sup> miševima na dijeti sa niskim sadržajem masti. Učestalost CD3<sup>+</sup>IL-17<sup>+</sup> i IL-5 pozitivnih mononuklearnih ćelija je bila značajno smanjena u VAT-u gojaznih ST2<sup>-/-</sup> miševa. Iako nije bilo razlike u serumskim nivoima IL-6 i CRP-a, koncentracija proinflamatornih citokina IL-1 $\beta$  i IFN- $\gamma$  je bila povećana u gojaznih ST2<sup>-/-</sup> miševa. Važno je istaći da su serumski nivoi anti-inflamatornih citokina, IL-10 i IL-13, bili niži u ST2<sup>-/-</sup> miševa u poređenju sa miševima divljeg soja.*

*Rezultati studije ukazuju na protektivnu ulogu IL-33/ST2 signalnog puta u pokretanju inflamacije u VAT-u nakon primene dijetete sa visokim sadržajem masti, koju karakteriše polarizacija makrofaga u pravcu M1 fenotipa i indukcija Th1 imunskog odgovora.*

**Ključne reči:** gojaznost, adipozno tkivo, inflamacija, citokini, makrofagi

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## ABBREVIATIONS

<b>BSA</b> -bovine serum albumin	<b>LFD</b> -low-fat diet
<b>CRP</b> -C reactive protein	<b>NKT</b> -natural killer T cells
<b>CD</b> -cluster of differentiation	<b>PBS</b> -phosphate-buffered saline
<b>FBS</b> -foetal bovine serum	<b>Th</b> -T helper cells
<b>FoxP3</b> - forkhead box P3	<b>TNF-<math>\alpha</math></b> -tumour necrosis factor- $\alpha$
<b>HFD</b> -high-fat diet	<b>WT</b> - wild-type
<b>IL</b> -interleukin	<b>VAT</b> -visceral adipose tissue
<b>IFN-<math>\gamma</math></b> -interferon- $\gamma$	

## INTRODUCTION

The complex pathogenesis of obesity and obesity-related metabolic disorders are strongly associated with a chronic low-grade inflammation that is characterised by an increased recruitment of immune cells into the visceral adipose tissue (VAT) [1]. Adipocytes are believed to play a central role in the initiation of the inflammatory response in response to metabolic danger signals during increased caloric intake [2]. The expanding adipose tissue found in obese individuals is predominantly infiltrated with IFN- $\gamma$ -producing Th1 and NKT cells, followed by an enhanced recruitment of classically activated M1 macrophages with a related decrease in the presence of alternatively activated M2 macrophages [3-6]. Activated macrophages produce pro-inflammatory cytokines, such as IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , which contribute to systemic inflammation and negatively impact insulin sensitivity [7]. This proinflammatory milieu resulting from nutrient excess is additionally characterised by a significantly decreased presence of immunosuppressive regulatory T cells in the visceral adipose tissue [8].

IL-33 is a newly identified member of the IL-1 cytokine family, which includes IL-1 and IL-18 [9]. Several lines of evidence suggest that IL-33 is a pleiotropic cytokine that signals through its receptor ST2 to orchestrate the innate and acquired immune responses [10]. Although IL-33 is primarily involved in the induction of Th2-type responses and can act directly on Th2 cells to increase the secretion of Th2 cytokines, such as IL-5 and IL-13, IL-33 can also promote Th1-type responses under certain conditions [11,12]. Additionally, IL-33 induces the production of pro-inflammatory cytokines and chemokines by mast cells and eosinophils and amplifies the polarisation of alternatively activated M2 macrophages [13].

IL-33 is a multifunctional cytokine involved in the pathogenesis of not only different inflammatory and autoimmune diseases, as well as in the pathogenesis of carcinogenesis [14-17]. Although IL-33 may play a protective role in obesity and atherosclerosis [18,19], the contribution of the IL-33/ST2 axis in metabolic disorders needs to be further elucidated. Aware that the BALB/c mice are relatively resistant to HFD-high-fat diet (HFD)-induced obesity, we investigated the role of ST2 in HFD-induced obesity using ST2 deficient (ST2<sup>-/-</sup>) and wild-type BALB/c mice.

## MATERIAL AND METHODS

### Animals

Six-week-old, male ST2 deficient (ST2<sup>-/-</sup>) and corresponding wild-type (WT) BALB/c mice were fed either a high-fat diet (HFD with 60% fat, obtained from Mucedola, Milan, Italy) or a low-fat diet (LFD with 3% fat, obtained from Mucedola, Milan, Italy) and were given free access to food and water. After 18 weeks on the specific diets, the animals were sacrificed, and the targeted tissues were collected for further examination. Blood collected from the abdominal aorta was centrifuged, and the isolated sera were stored at -20°C until further analysis. All animal procedures were approved by the Ethical Committee of the Faculty of Medical Sciences at the University of Kragujevac.

### Metabolic parameters

Body weight and fasting blood glucose levels were measured every second week of the month. To evaluate forest for glycaemia, whole blood was collected via tail vein puncture and assessed using the Accu-Chek glucometer (Roche Diagnostics, Mannheim, Germany). The total visceral adipose tissue was isolated from the peritoneal cavity and measured after sacrifice.

### Isolation of mononuclear cells from the visceral adipose tissue

The visceral adipose tissue was minced and washed twice in PBS containing 10% FBS. The tissue was then digested with 1 mg/ml collagenase type II (Sigma-Aldrich, St. Louis, MO, USA) in PBS containing 2% BSA for 1 h at 37°C with vigorous shaking. The digested tissue was passed through a 40  $\mu$ m nylon cell strainer (BD Biosciences, San Jose, CA, USA), and the red blood cells were lysed using an erythrocyte lysis buffer. The isolated cells were then washed twice and resuspended in a RPMI cell medium (Sigma-Aldrich) containing 10% FBS for flow cytometric analysis.

### Flow cytometry

The cells were labelled with the following fluorochrome-conjugated monoclonal antibodies: anti-mouse CD3, CD4, IL-17, IL-5, CD25, FoxP3, F4/80, CD206 and CD11c (all from BD Biosciences). For intracellular staining, the cells were activated using PMA (50 ng/ml) and ionomycin (500 ng/ml) (Sigma-Aldrich) with GolgiStop (BD Biosciences)



for 5 h at 37°C and then stained with the fluorochrome-conjugated antibodies using the Cytotfix/Cytoperm kit (BD Biosciences) according to the manufacturer's protocol. The cells were analysed using a FACS Calibur flow cytometer (BD Biosciences), and the analysis was conducted with the FlowJo software (Tree Star).

### Serum cytokines measurement

The sera were assayed for CRP, IL-1 $\beta$ , IL-6, IFN- $\gamma$ , IL-10 and IL-13 using highly sensitive enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) that were specific for these mouse cytokines; the kits were used in accordance with the manufacturer's instructions.

### Statistical analysis

All data are presented as the mean  $\pm$  SE. The data were analysed with the statistical package SPSS, version 13, by using either a two-tailed Student's t-test or the nonparametric Mann-Whitney test, where appropriate. The results were considered significantly different when  $p < 0.05$ .

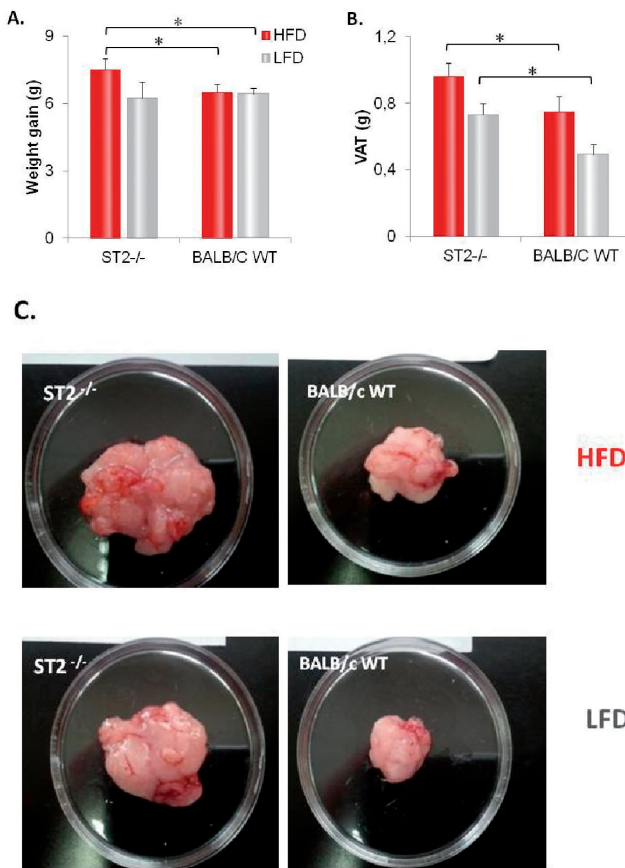
## RESULTS

### Deletion of ST2 accelerates HFD-induced adiposity

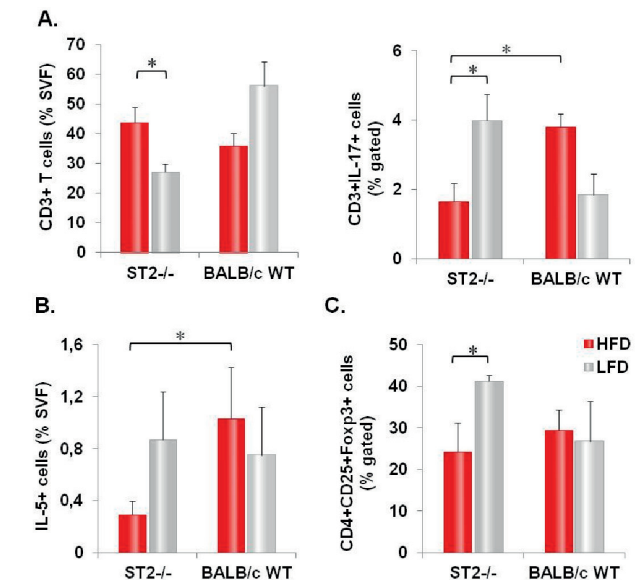
After 18 weeks on the specific diets, we observed a significant weight gain in the HFD-fed ST2<sup>-/-</sup> mice compared to the WT mice on both diet regimes. Total weight gain was determined as the difference in body weight of each mouse after 18 weeks on a specific diet and the body weight measured on day 0. The body weight did not differ among the groups on day 0 (data not shown). Our data showed that the HFD-fed ST2 deficient mice had a significantly increased total weight gain during the 18 weeks when compared to both the HFD-fed and the LFD-fed WT mice (Figure 1A). We also observed a significantly larger amount of total visceral adipose tissue in the HFD-fed ST2<sup>-/-</sup> mice than in the corresponding WT animals (Figure 1B, 1C). At the same time, the amount of total visceral adipose tissue isolated from the LFD-fed ST2 deficient mice was significantly higher than that isolated from the LFD-fed WT mice, indicating the relevance of the ST2 molecule in the expansion of the visceral adipose tissue (Figure 1B, 1C).

### The adipose tissue of obese ST2<sup>-/-</sup> mice have an increased percentage of CD3<sup>+</sup> T cells, fewer CD3<sup>+</sup>IL-17<sup>+</sup> and IL-5 expressing mononuclear cells and decreased regulatory T cells than the adipose tissue of ST2<sup>-/-</sup> lean mice

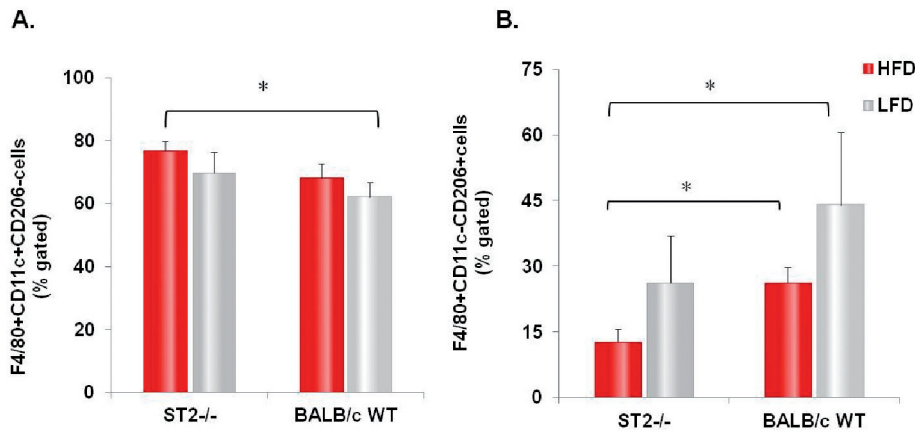
Flow cytometric analysis of mononuclear cells isolated from the visceral adipose tissue showed that the HFD increased the infiltration of CD3<sup>+</sup> T cells into the visceral adipose tissue of the ST2 deficient mice, and these cells expressed lower levels of IL-17 than those of the HFD-fed WT mice and



**Figure 1. Increased visceral adiposity in HFD-fed ST2 deficient mice.** A. Total weight gain was individually determined for each mouse after 18 weeks on the specific diet regimens. B. Total visceral adipose tissue was excised and measured after sacrifice, which occurred 18 weeks after the mice began the specific diet regimens. C. Representative photographs of isolated visceral adipose tissue after sacrifice. The data are presented as the mean  $\pm$  SE. Statistical significance was determined at \* $p < 0.05$ .



**Figure 2. A higher percentage of T cells and a decreased presence of IL-17-producing CD3<sup>+</sup> cells, IL-5-positive mononuclear cells and regulatory T cells in the VAT of HFD-fed ST2<sup>-/-</sup> mice.** Mononuclear cells isolated from visceral adipose tissue were labelled with fluorochrome-conjugated anti-mouse antibodies and analysed with flow cytometry. The presence of different cell phenotypes was determined as a percentage of the gated mononuclear cells. The data are presented as the mean  $\pm$  SE. Statistical significance was determined at \* $p < 0.05$ .



**Figure 3. A decreased percentage of alternatively activated M2 macrophages in the HFD-fed ST2<sup>-/-</sup> mice.** The frequencies of different cell phenotypes are presented as percentages of the gated mononuclear cells isolated from visceral adipose tissue using a collagenase digestion protocol. The isolated cells were labelled with fluorochrome-conjugated anti-mouse antibodies and analysed using flow cytometry. The data are presented as the mean ± SE. Statistical significance was determined at \*p<0.05.

the LFD-fed ST2<sup>-/-</sup> mice (Figure 2A). The number of IL-5-producing mononuclear cells was significantly decreased in the visceral adipose tissue of the HFD-fed ST2<sup>-/-</sup> mice compared to the number in the corresponding WT animals (Figure 2B). The presence of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells in the visceral adipose tissue was significantly reduced in the ST2 deficient mice after HFD feeding (Figure 2C).

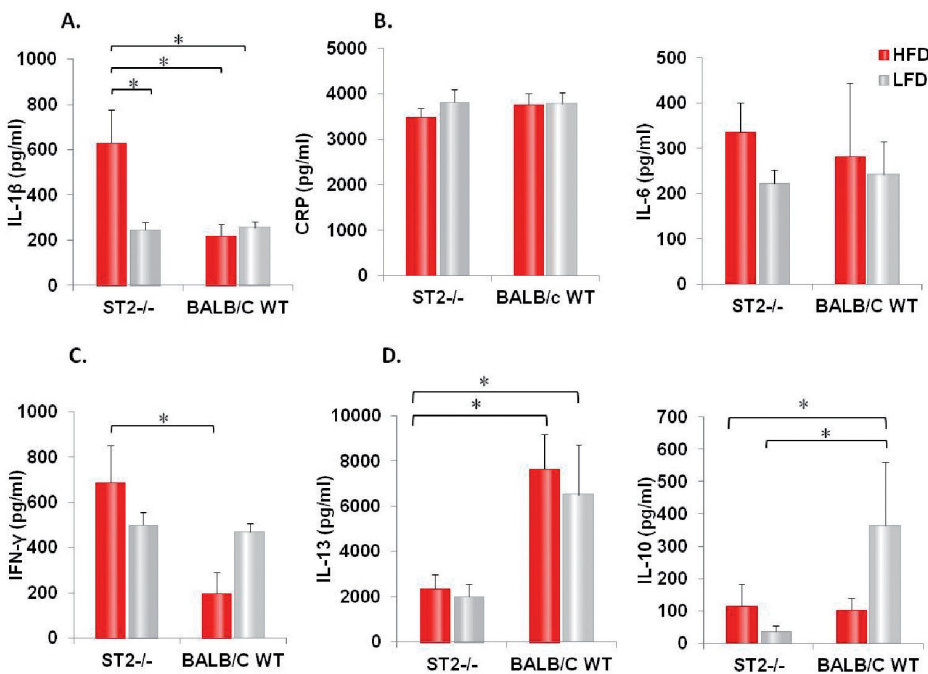
### Markedly reduced alternatively activated M2 macrophages in the VAT of obese ST2<sup>-/-</sup> mice

To further understand obesity-related inflammation in the studied mice after 18 weeks, we investigated the recruitment of macrophages into the visceral adipose tissue and analysed their phenotypes. The number of the proinflammatory F4/80<sup>+</sup>CD11c<sup>+</sup>CD206<sup>+</sup> macrophages was significantly increased in the HFD-fed ST2 deficient mice when compared

with the LFD-fed WT mice (Figure 3A), but this number was not increased compared to the other experimental groups. However, the number of alternatively activated F4/80<sup>+</sup>CD11c<sup>+</sup>CD206<sup>+</sup> M2 macrophages was markedly reduced in the visceral adipose tissue of the HFD-fed ST2<sup>-/-</sup> mice in comparison with both the diet-matched WT animals and the LFD-fed WT mice (Figure 3B).

### Obese ST2<sup>-/-</sup> mice have increased serum levels of the proinflammatory IL-1β and IFN-γ and lower levels of the anti-inflammatory IL-13 and IL-10

The systemic inflammatory profile of the experimental mice was evaluated by measuring the serum cytokine levels. After 18 weeks on a HFD, the ST2 deficient mice had exhibited markedly elevated levels of the proinflammatory cytokine IL-1β than compared to the diet-matched WT mice and the LFD-fed mice of both genotypes (Figure 4A). At the same time, we did not observe any difference in the serum levels of the C-reactive protein (CRP) and IL-6 among the experimental groups (Figure 4B). However, the serum level of IFN-γ was significantly increased in the HFD-fed ST2<sup>-/-</sup> mice than compared to in the corresponding WT mice (Figure 4C). After 18 weeks on the specific diet, both the ST2 deficient mice on the HFD and LFD had a significantly lower systemic levels of IL-13 when compared to the WT mice on the respective diets; additionally, both the HFD-fed and LFD-fed ST2 deficient mice exhibited decreased production of the anti-inflammatory IL-10 than compared to the LFD-fed WT mice (Figure 4D).



**Figure 4. Increased levels of the proinflammatory cytokines IL-1β and IFN-γ and decreased levels of the anti-inflammatory cytokines IL-13 and IL-10 in the sera of obese ST2 deficient mice.** The levels of cytokines in the serum were measured for each mouse after 18 weeks on the different diet regimens using specific ELISA tests. The data are presented as the mean ± SE. Statistical significance was determined at \*p<0.05.



## DISCUSSION

In this study, we showed that the ablation of ST2 enhances the visceral adiposity of HFD-fed mice, as indicated by a significant increase in weight and a growing amount of visceral adipose tissue. The amount of VAT was significantly increased in both the HFD-fed ST2<sup>-/-</sup> mice and the LFD-fed ST2<sup>-/-</sup> mice compared to their diet-matched WT counterparts. The enhanced adiposity of the ST2 deficient mice was characterised by an increased presence of CD3<sup>+</sup> T cells, which is in line with previously reported data showing that the infiltration of T cells into the visceral adipose tissue and their polarisation toward a Th1 phenotype played a crucial role in high-fat diet-induced obesity [3,20]. The induction of Th2 cytokine production is a well-established result of the interaction between IL-33 and the ST2 receptor [11]. Multiple cell types, including the adipocytes in the visceral adipose tissue, can most likely produce IL-33, resulting into the maintenance of tissue homeostasis by promoting a Th2 immune response and the production of Th2 cytokines, such as IL-4, IL-5 and IL-13 [21]. According to those findings, a HFD resulted in a significantly decreased the percentage of IL-5 positive mononuclear cells in the VAT of ST2-deficient mice compared to the HFD-fed WT mice. Interestingly, our data showed a significantly lower incidence of IL-17-producing CD3<sup>+</sup> cells in the HFD-fed ST2 knockout mice in contrast to the corresponding WT mice and the LFD-fed ST2 deficient mice. Although there is evidence that IL-17 may be a negative regulator of adipose tissue inflammation [22], the decreased expression of IL-17 could be related to the enhanced Th1 immune response during obesity [23].

There is evidence that regulatory T cells play an important role in the maintenance of adipose tissue homeostasis and glucose sensitivity [24]. During diet-induced inflammation, the presence of T regulatory cells in the visceral adipose tissue decreases [8], which is in line with our findings that a HFD significantly reduced the incidence of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells in the ST2 deficient mice.

The polarisation of infiltrated macrophages toward the classically activated M1 phenotype and a significant reduction in the amount of alternatively activated M2 macrophages is the mechanism underlies the diet-induced inflammation in the visceral adipose tissue [6]. However, IL-33 promotes the phenotypic switch of macrophages to an M2 phenotype during obesity [18]. We found that the lack of the IL-33 receptor ST2 expression is correlated with the markedly increased presence of the F4/80<sup>+</sup>CD11c<sup>+</sup>CD206<sup>+</sup> M1 macrophages in the visceral adipose tissue of the HFD-fed mice. These M1 macrophages were recently described as the proinflammatory cell subset present during diet-induced obesity [25]. At the same time, we found a significantly decreased incidence of the alternatively activated F4/80<sup>+</sup>CD11c<sup>+</sup>CD206<sup>+</sup> M2 macrophages, which is in line with a previous report related to the phenotypic switch of macrophages in diet-induced obesity in mice [26].

Accelerated HFD-induced adiposity in the absence of ST2 was associated with increased systemic levels of proinflammatory cytokines, such as IL-1 $\beta$  and IL-6 [7], and decreased levels of the anti-inflammatory cytokines IL-13 and IL-10 [27, 28]. Although we did not find any differences in the production of IL-6 and CRP between the experimental groups, the systemic level of the proinflammatory IL-1 $\beta$  was significantly increased in the HFD-fed ST2 deficient mice than in the other experimental groups. In the absence of IL33/ST2 signalling, we observed significantly decreased levels of the anti-inflammatory cytokines IL-13 and IL-10, which were strongly correlated with the enhanced obesity in the ST2 deficient mice.

## CONCLUSIONS

These findings suggest that IL33/ST2 signalling plays an important protective role in high-fat diet-induced adipose tissue inflammation and could be of therapeutic relevance.

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No potential conflicts of interest relevant to this article were reported.

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