STAT4 regulates CD8+Treg/Tfh cell axis and promotes atherogenesis in insulin-resistant Ldlr−/− mice

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Abstract

The metabolic syndrome and diabetic conditions support atherosclerosis, but the exact mechanisms for accelerated atherogenesis remain unclear. While the pro-inflammatory role of signal transducer and activator of transcription 4 (STAT4) in atherosclerosis and diet-induced insulin resistance (IR) was recently established, an impact of STAT4 on atherogenesis in conditions of IR is not known. Here we generated Stat4−/−Ldlr−/− mice that were fed a diabetogenic diet with added cholesterol (DDC). DDC fed Stat4−/−Ldlr−/− mice demonstrated improved glucose tolerance, insulin sensitivity, and a 36% reduction in atherosclerosis compared with Ldlr−/− controls. Interestingly, we detected a reduction in T follicular helper (Tfh) and plasma B cells, but a sharp elevation in CD8+ Tregs in spleens and aortas of Stat4−/−Ldlr−/− versus Ldlr−/− mice. Similarly, STAT4 deficiency supported CD8+ Treg differentiation in vitro. Stat4-deficient CD8+ Tregs suppressed Tfh and germinal center B cell development upon immunization with KLH indicating an important role for STAT4 in CD8+ Treg functions in vivo. Furthermore, adoptive transfer of Stat4−/−Ldlr−/− CD8+ Tregs vs Ldlr−/− mice resulted in a significant reduction of plaque burden, suppression of Tfh and germinal center B cells in DDC fed Ldlr−/− recipients. STAT4 expression in macrophages also affected the Tfh/CD8+Treg axis, as conditioned media from Stat4−/−Ldlr−/− MΦs supported CD8+ Treg but not Tfh cell differentiation in TGFβ-dependent manner. These findings suggest a novel mechanism by which STAT4 supports atherosclerosis in IR Ldlr−/− mice via STAT4-dependent macrophage as well as cell-intrinsic

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suppression of CD8+ Treg generation and functions and maintenance of Tfh cell generation and accompanied humoral immune response.

Keywords
Atherosclerosis; Insulin Resistance; Inflammation; Leukocytes; Transcription factors

Introduction
Atherosclerosis is a multifactorial chronic inflammatory disease characterized by the accumulation of modified lipoproteins and immune cells in the aortic wall, vascular dysfunction, and low-grade chronic inflammation. Atherosclerosis is the prominent cause of cardiovascular diseases and mortality in many countries. Inflammation impacts all stages of atherogenesis and is a common denominator between atherosclerosis, obesity, insulin resistance (IR), and type 2 diabetes. While the role of IR in atherosclerosis is recognized, there are limited numbers of murine models that can be used to investigate simultaneous effects of IR and inflammation on the induction of atherosclerosis. Low-density lipoprotein receptor (Ldlr−/−) deficient mice develop obesity, IR, and atherosclerosis when fed a diabetogenic diet with added cholesterol (DDC). Importantly, this study showed that male mice fed DDC diet not only have increased macrophage (MΦ) infiltration and inflammation in adipose tissues, but also significantly increased MΦ content in the aorta within atherosclerotic plaques and accelerated atherogenesis. Therefore, we chose to use this model of diet-induced obesity and IR to investigate a role of signal transducer and activator of transcription 4 (STAT4) in IR-accelerated atherosclerosis.

STAT4 is an important regulator of inflammatory immunity and autoimmune-related inflammation. The activation of STAT4 is regulated by the pro-inflammatory cytokine IL-12 and IL-23 released mainly by antigen presenting cells. STAT4 and the T-box transcription factor (T-bet) are key transcription factors that support the differentiation of Th1 cells. Importantly, the expression of STAT4 also suppresses the expansion of Foxp3+CD4+ Tregs. Thus STAT4 is involved in the regulation of a delicate balance between pro-inflammatory and suppression arms of the immune response. Recent work on the biology of STAT4 has revealed that STAT4 is not exclusively expressed by T and NK cells, but can be also detected in IFNα or LPS-stimulated monocytes and dendritic cells. Although the role of STAT4 in myeloid cells is not well-understood, there is some evidence suggesting a key role of STAT4 in MΦ biology. Attenuated microbicidal activity, reduced nitric oxide NO and IFNγ levels were detected in STAT4-deficient MΦs. Our data also demonstrated reduced cytokine production, low activation, diminished expression of CCR2 in Stat4-deficient M1 or M2 MΦs.

While several subsets of T cells and MΦs play an important role in atherosclerosis, additional leukocyte populations including T follicular cells (Tfh) and CD8+ Tregs are now reported to have a role in this disease. Tfh cells assist in the induction of germinal centers, and support the activation and differentiation of memory B cells and plasma cells regulating the generation of antigen-specific antibodies. Importantly, Clement and colleagues...
demonstrated that Tfh cells support atherogenesis via the production of pathological antibodies and generation of highly active germinal centers\textsuperscript{13}. The authors also showed that CD8\(^{+}\)Tregs control Tfh cell development and formation of follicular helper-germinal center B-cells during atherogenesis. The IL-12/STAT4 pathway is involved in the gene expression of \textit{Il21} and \textit{Bcl6}, both of which are necessary for the generation of Tfh cells\textsuperscript{14–17}. In line with this observation, it is reported that the \textit{in vitro} differentiation of human Tfh cells is supported by STAT3/STAT4 signaling\textsuperscript{18}. However, the role of STAT4 in generation of Tfh cells under atherosclerosis-prone conditions has not been examined.

Mounting evidence has demonstrated that a population of CD8\(^{+}\)CD122\(^{+}\) Tregs controls the generation of autoreactive CD4\(^{+}\) T cells as well as formation of Tfh cells\textsuperscript{19,20} suppressing both autoimmune and alloimmune responses. Importantly, in atherosclerosis-prone conditions, CD8\(^{+}\) Treg cells suppress the development of Tfh cells and formation of germinal centers in \textit{Apoe}\(^{-/-}\) mice\textsuperscript{13}. While the functions of CD8\(^{+}\) Tregs are currently under active investigation, the transcriptional network that controls differentiation of CD8\(^{+}\) Treg is unknown. In this study, we demonstrate that STAT4 suppresses CD8\(^{+}\)Treg functions and affects a well-known ability of CD8\(^{+}\) Treg to defeat generation of Tfh and germinal B cells in vivo. Additionally, STAT4 also supports MΦ activation and modulation of the pro-inflammatory immune composition within the aorta. The results obtained in this study could lead into novel drug therapy using inhibitors against STAT4 to regulate both the immune response and IR-related inflammation in order to provide a duel-strategy to combat IR-associated atherogenesis.

**Materials and Methods**

**Animals**

\textit{Stat4}\(^{-/-}\) mice\textsuperscript{21} were crossbred with \textit{Ldlr}\(^{-/-}\) mice (Jackson Labs, Bar Harbor, ME) to generate \textit{Stat4}\(^{-/-}\textit{Ldlr}^{-/-}\) mice. For some experiments C57BL/6 and \textit{Stat4}\(^{-/-}\) mice were used. Beginning at 8 weeks of age, male \textit{Stat4}\(^{-/-}\textit{Ldlr}^{-/-}\) and \textit{Ldlr}\(^{-/-}\) mice were fed a diabetogenic diet with added cholesterol (DDC) diet (BioServ, protein 20.5%, fat 36.0%, carbohydrates 35.7%, cholesterol 0.15%, #S6524) for 11 or 16 or 24 weeks. All animals were kept in specific pathogen-free conditions, and animal experiments were approved by the Eastern Virginia Medical School Animal Care and Use Committee.

**Quantification of Atherosclerosis**

The aortas of \textit{Stat4}\(^{-/-}\textit{Ldlr}^{-/-}\) and \textit{Ldlr}\(^{-/-}\) mice were collected and stained with Oil Red O (ORO), then microdissected longitudinally and pinned as described earlier. Images were scanned and the surface area percentage occupied by lesions was determined by two independent investigators with ImageJ (NIH). Hearts were harvested then fixed with 4% PFA via cardiac puncture. From the point of the appearance of aortic valve leaflets, sequential 5 μm thick sections were cut and six sections over 300 μm distance were collected, and analyzed by Russell modified Movat staining as previously described\textsuperscript{12}. Total cholesterol and triglyceride levels were determined according to the manufacturer’s instructions.
Flow cytometry analysis of immune cells within aorta, spleen, and PLN

Single cell suspensions from the aorta were prepared as previously described\textsuperscript{12,22}. Briefly, mice were anesthetized using CO\textsubscript{2}, blood was collected via cardiac puncture. Next, the heart was perfused with PBS containing 20 U/ml of heparin by cardiac puncture. Aortas were then microdissected and enzymatically digested for 1 hour at 37°C with 125 U/ml Collagenase type XI, 60 U/ml hyaluronidase type I-s, 60 U/ml DNase1 and 450 U/ml Collagenase type I (Sigma-Aldrich, St. Louis, MO) in PBS as described previously\textsuperscript{12,22}. Aortas, spleens, and para-aortic lymph node (para-aortic LN) and peripheral LN (PLN), were rubbed in a 70μm cell sieve (Corning Incorporated Life Sciences, Tewksbury, MA). Erythrocytes in spleens were lysed using ACK lysis buffer (8.29mg/ml NH\textsubscript{4}CL, 1mg/ml KHCO\textsubscript{3}, 0.372mg/ml EDTA, all from Sigma-Aldrich). Cell numbers were determined using trypan blue (MP Biomedicals, LLC, Solon, OH) and the hemocytometer. Intracellular staining for Tbet, Fox3p, CD68, and Bcl6 was performed according to the Fix&Perm® cell permeabilization protocol (BD Biosciences, San Jose, CA). The Cytek DXP8 Color (Cytek Development Inc.) upgraded FACSCalibur™ (BD Biosciences, San Jose, CA) was used to collect samples and data analysis was conducted with FlowJo (Tree Star Inc., Ashland, OR). In all flow cytometry experiments isotype control and fluorescent minus one control were used to set appropriate gating for the samples. To exclude doublets from analysis, a forward scatter-area against forward scatter-linear gate was used.

Dyes, recombinant proteins and antibodies

The antibodies used were as follows: CD19-PECy7 (1D3), CD8-APC (53-6.7), Ly6C-FITC (AL-21), CD68-PE (FA11), CD44-FITC (IM7), CD45-PerCP (30-F11), CD69-PE (H1.2F3), CXCR5-eFluor50 (SPRCL5), CD138-APC (281-2), CD21-PE (7G6), CD275-PE (HK5.3), CD122-APC (TM-b1), CD11b-PE (M1/70.15), IFN\textgreek{g}eFluor 450 (XMG1.2), CD4-PerCP (L3T4), F4/80-APC-eFluor780 (BM8), Foxp3-PE (MF23), CD3-APC-CY7/eFluor780 (17A2), Ly-6G-PE (1A8), from eBioscience (San Diego, CA) and anti-mouse CD16/CD32 (The Lymphocyte Culture Centre, UV A, Charlottesville, VA). For some staining, we used: PerCP-CD4, biotinylated-CXCR5, followed by APC-streptavidin, or FITC-GL-7, PE-FAS and PerCP-B220 staining (all from Biologend). To distinguish between live and dead cells, Viability Live Dead-eFluor50 (eBioscience, San Diego, CA) was used. Anti–CD3 and –CD28 were utilized for in vitro assays (eBioscience, San Diego, CA). Recombinant proteins used were as followed: mouse TGF\textgreek{B} and IL-2 was purchased from PeproTech (Rocky Hill, NJ). LPS was purchased through Sigma-Aldrich (St. Louis, MO).

Quantitative real time PCR

Total RNA was extracted from splenic cells and peritoneal MΦs using Trizol® reagent (Invitrogen™, Life Technologies, Grand Island, NY). DNase I treatment was used to remove contaminating genomic DNA (Qiagen, Germantown, MD). Approximately 1 μg of total RNA was reverse transcribed to cDNA by synthesis reaction using Promega reverse transcription kit: containing reverse transcription 10x buffer without MgCl\textsubscript{2}, MgCl\textsubscript{2}, random primers, 10 mM dNTPs mix, AMV Reverse Transcriptase (HC), and Recombinant RNasin® Ribonuclease. Real time PCR was performed using Taqman probes from Applied Biosystems (Carlsbad, CA), 10 mM dNTPs, 10x PCR buffer without MgCl\textsubscript{2}, MgCl\textsubscript{2}, and
Jumpstart Taq polymerase (Sigma-Aldrich, St. Louis, MO) for iNos, Mrc1, Il21, Il27 for splenic cells, and Ifnγ, Il6, Tgfβ, and Il27 for LPS stimulated peritoneal Φ. Ct values for cDNA were determined using a CFX96™ Real-Time System C1000™ Thermal Cycler detection system (Bio-Rad laboratories). The results were normalized to housekeeping gene 18S.

**CD8+ Treg cell Differentiation in vitro**

Briefly, CD8+ T cells from Stat4−/−Ldlr−/− and Ldlr−/− were isolated using Stem Cell kit protocol (EasySep™ Mouse CD8+ T Cell Isolation Kit, STEMCELL Technologies, Canada) and then plated with rhTGFβ (2 ng/mL), rhIL-2 (100 U/mL), plate-bound anti-CD3 (1 μg/mL), and soluble anti-CD28 (1 μg/mL) in complete RPMI-1640 supplemented media with 10% FBS, 1% Penicillin/Streptomycin, 1% Glutamax, 1% Hepes, 0.5% NEAA, 0.5% Sodium pyruvate, and 50 μM BME) for 3 days. Then FACS analysis was used to identify differentiated CD8+ Tregs as CD8+CD122+ cells.23

**Impact of media isolated from LPS-activated peritoneal Mφs on Th cell and CD8+ Treg differentiation**

Briefly, peritoneal Mφs from Stat4−/−Ldlr−/− and Ldlr−/− were plated in complete RPMI-1640 supplemented media with 10% FBS, 1% penicillin/streptomycin, 1% glutamax, 1% Hepes, 0.5% NEAA, 0.5% sodium pyruvate, and 50 μM BME for 2 hours. Thioglycollate was not used for the isolation of peritoneal Mφs. Lymphocytes were removed by washing with complete media. Next, LPS (500 ng/mL) supplemented completed media was used to activate peritoneal Mφs for 48 hours then the conditioned media and the peritoneal Mφs were collected. Ldlr−/− splenic CD4+ or CD8+ T cells were isolated using the Stem Cell Kit protocol (STEMCELL Technologies, Canada), and plated with immobilized anti-CD3, soluble anti-CD28 and the conditioned media from the Stat4−/−Ldlr−/− and Ldlr−/− LPS activated peritoneal Mφs. Differentiation into T helper cell types or CD8+CD122+ Treg was determined 48 hours later using flow cytometry.

**Glucose Tolerance Test and Insulin Tolerance Test**

To perform glucose tolerance tests (GTT), fasted overnight mice were injected intraperitoneally with filter-sterilized 2 g/kg glucose in 0.9% NaCl. A tail vein blood sample was taken before the injection and at 10, 20, 30, 60, 90, and 120 min after injection for measurements of blood glucose levels. The insulin tolerance test (ITT) was conducted by intraperitoneally injection with insulin (0.75 U/kg) in 0.9% NaCl. A tail vein blood sample was taken immediately before and at 15, 30, 45, and 60 min after the injection for analysis of blood glucose levels. GTT and ITT were performed on the same set of mice that were used for en face analysis.

**Adoptive-Transfer of CD8+ Tregs and immunization**

CD8+CD122+ STAT4-sufficient or STAT-4-deficient Treg cells (CD45.2+) were intravenously transferred into C57BL/6 (CD45.1+) mice (0.5×10^6 cells/recipient, n=4/per group). A group of C57BL/6 mice that did not receive T cells was used as a control. All mice were immunized subcutaneously with 1000 μg KLH emulsified in CFA as we
described before. Seven days after immunization, draining lymph nodes from the recipients were collected; cell suspensions were prepared and stained with the antibodies as follows: PE-Bcl6, PerCP-CD4, biotinylated-CXCR5, followed by APC-streptavidin, or FITC-GL-7, PE-Fas and PerCP-B220, and analyzed by flow cytometry. Antigen-specific IgM, IgG and IgA Abs in sera from immunized mice were measured with ELISA. In brief, serum samples were added in a 3-fold serial dilution onto plates pre-coated with 10 μg/ml KLH. Antigen-specific Abs were detected with biotinylated goat anti-mouse IgM, IgA or rat anti-mouse IgG Abs (Southern Biotechnology Associates).

Adoptive-Transfer of CD8+ Tregs into Ldlr−/− recipients

Sorted CD8+CD122+ Stat4−/−Ldlr−/− or CD8+CD122+ Ldlr−/− cells were intravenously transferred into 8 week Ldlr−/− mice (0.9×10^6 cells/recipient, n=5/per group). For basal levels of plaque burden, a group of Ldlr−/− mice (n=6) was injected with PBS. After 11 weeks of DDC feeding, aortas were analyzed for plaque burden using Oil Red O. Additionally, aortas, spleens, blood, para-aortic LN and PLN were collected from Ldlr−/− recipients that received CD8+CD122+ Stat4−/−Ldlr−/− or CD8+CD122+ Ldlr−/− donor cells. Cell suspensions from PLN, para-aortic LN, and spleen were prepared, stained for Thf and germinal center B cells, and analyzed by flow cytometry. Obtained plasma was analyzed for the presence of IgM, IgG1, IgG2a, IgG2b, IgG k, IgG λ, IgG3, and IgA using a Mouse Immunoglobulin Isotyping ELISA (BD Bioscience).

Statistical Analysis

Data were analyzed by Graphpad Prism6, comparisons were made using Student’s or Mann-Whitney test with the data expressed as mean±SEM. Comparisons of three or more groups were conducted using an ANOVA and multiple comparisons using TUKEY test. Statistical significance was set at p<0.05.

Results

STAT4-deficiency attenuates atherosclerosis and improves metabolic parameters in Stat4−/−Ldlr−/− mice fed a diabetogenic diet with added cholesterol

We recently demonstrated a pro-atherogenic role of STAT4 in Apoe−/− mouse model of atherosclerosis; however, the role of STAT4 in IR-accelerated atherogenesis remains undefined. Schreyer et al. reported that the consumption of diabetogenic diet with added cholesterol (DDC) results in obesity, insulin resistance, and accelerated atherogenesis in Ldlr−/− mice. Therefore, to investigate the role of STAT4 in IR-accelerated atherosclerosis, we generated Stat4-deficient Ldlr−/− (Stat4−/−Ldlr−/−) mice and used DDC diet for the course of this study. In line with the report from Schreyer et al. we detected impaired glucose tolerance in the glucose tolerance test (GTT, Fig. 1A) and diminished insulin sensitivity in the insulin tolerance test (ITT, Fig. 1B) in Ldlr−/− mice fed DDC in comparison with CD fed Ldlr−/− mice. These results using the area under the curve (AUC) of DDC vs CD fed mice indicate that DDC feeding results in glucose intolerance and IR in Ldlr−/− mice. Next we investigated the impact of STAT4 deficiency on the development of IR in DDC fed Stat4−/−Ldlr−/− mice. DDC fed Stat4−/−Ldlr−/− mice displayed reduced area...
under the curve (AUC) in the insulin and glucose tolerance tests, indicating improved insulin sensitivity and glucose homeostasis (Fig. 1A–B) compared to diet-matched Ldlr−/− mice.

To directly assess the impact of STAT4 deficiency on atherogenesis, we examined the plaque burden between 16 week DDC fed Stat4−/−Ldlr−/− and Ldlr−/− male mice. At 16 weeks DDC feeding, male Stat4−/−Ldlr−/− mice had a 36% reduction in aortic lesions in comparison to age- and diet-matched Ldlr−/− control mice (5.8±0.6% and 9.1±1.2%, respectively; p<0.02; Fig. 1C). Moreover, there was a reduction in cross-sectional plaque area in the aortic roots of 16 week DDC fed Stat4−/−Ldlr−/− in comparison with Ldlr−/− mice (Fig. 1D). Diet and age matched Stat4−/−Ldlr−/− and Ldlr−/− mice display no difference in body weight, plasma cholesterol, and triglyceride when fed a DDC diet for 16 weeks (data not shown). To further examine the role of STAT4 in the regulation of atherosclerosis at the advanced stage of atherogenesis, we next analyzed Stat4−/−Ldlr−/− mice that were fed DDC diet for 24 weeks. STAT4 deletion resulted in ~45% reduction in plaque burden in Stat4−/−Ldlr−/− mice in comparison with Ldlr−/− controls (9.6±2.0% and 17.4±1.2%, respectively; p<0.005; Fig. 1E). Altogether these results clearly demonstrate a prominent pro-atherogenic role for STAT4 at the different stages of IR-accelerated atherogenesis in DDC fed Ldlr−/− mice.

Stat4-deficiency reduces Tfh cell content, but supports the development of CD8+Treg in spleens of Stat4−/−Ldlr−/− mice

Atherosclerosis-prone conditions induce differentiation of Tfh cells and increased germinal center formation with a concomitant elevation in plasma levels of immunoglobulins in aged Apoe−/− mice13. To get insight into a role of STAT4 in the regulation of Tfh cell content in atherogenesis, we first analyzed the cellularity and immune composition of secondary lymphoid organs in Stat4−/−Ldlr−/− and Ldlr−/− mice fed DDC diet. We found a reduced number of CD4+ cells in spleens of Stat4−/−Ldlr−/− versus Ldlr−/− mice (Fig. 2A). Interestingly, reduced expression of CD69, as early activation marker, and CD44, as a marker of a long-term memory phenotype was detected in splenic CD4+ T cells from Stat4−/−Ldlr−/− mice in comparison with Ldlr−/− mice (Fig. 2B) suggesting at least to some extent a defective T cell activation under the conditions of STAT4-deficiency. Importantly, Stat4−/− mice have no difference in splenic T and B cell composition in comparison with the spleen of C57BL/6 mice under normal/non-inflamed conditions21,25. In agreement with these data, we also found no difference in the percentage of splenic CD4+ and CD8+ T cells between Stat4−/− and BL/6 mice ((CD4+: 21.4%±0.8% and 21.7%±0.9%, CD8+: 15.1%±0.8% and 13.7%±0.6% for Stat4−/− and C57BL/6, respectively). Thus, the reduction in splenic CD4+ cells in Stat4−/−Ldlr−/− mice is likely due to specific effects of STAT4 deficiency under inflammatory conditions of atherosclerosis.

Tfh cells are detected in spleens and tertiary lymphoid structures at the advanced stages of atherosclerosis13. To determine if STAT4 plays a role in Tfh cell content under DDC diet induced-atherosclerotic conditions, we examined the relative proportion and number of Tfh cells in the spleen using the expression of Tfh cell-defining chemokine receptor, (C-X-C) receptor 5 (CXCR5). STAT4-deficiency caused a significant reduction in the cell numbers of splenic CD4+CXCR5+ Tfh cells in Stat4−/−Ldlr−/− mice in comparison with Ldlr−/− spleens (1.1±0.2×10^6 vs 2.2±0.6×10^6 respectively, p<0.05; Fig. 2C). One of the major roles of Tfh
cells is to regulate the differentiation of B cells to plasma B cells, resulting in the generation of specific antibody responses\(^{26}\). Next, we sought to determine whether reduced numbers of Tfh cells in the spleen of Stat4\(^{-/-}\)Ldlr\(^{-/-}\) mice would affect plasma B cell content under the conditions of STAT4 deficiency. There was a significant reduction in the number of CD19\(^{+}\)CXCR5\(^{-}\)CD138\(^{+}\)CD21\(^{-}\) plasma B cells in Stat4\(^{-/-}\)Ldlr\(^{-/-}\) mice compared to Ldlr\(^{-/-}\) controls (Fig. 2E–F).

CD8\(^{+}\) Tregs are important suppressors of T cells that control the development of autoreactive CD4\(^{+}\) T cells. CD8\(^{+}\) Tregs also target Tfh cells because of their high basal expression of the Qa-1 molecule and thus indirectly regulate the formation of germinal centers, heavy-chain class switching, and affinity maturation of antibodies\(^{20}\). CD8\(^{+}\) Tregs are typically identified as CD8\(^{+}\)CXCR5\(^{-}\)CD275\(^{+}\)CD122\(^{+}\) cells and found in small numbers in secondary lymphoid tissues\(^{20}\). An example of the gating scheme used for analysis is depicted in Fig. 3A. Interestingly, a recent study demonstrated a protective role of splenic CD8\(^{+}\) Tregs in atherosclerosis via the down-regulation of Tfh cell number and reduced generation of germinal center formation and plasma B-cells\(^{13}\). While the transcriptional network that is responsible for the induction of CD4\(^{+}\) Tregs is well-defined, transcriptional factors that are responsible for the induction/maintenance of CD8\(^{+}\) Tregs remain elusive. Since we found reduced numbers of Tfh cells in spleens of Stat4\(^{-/-}\)Ldlr\(^{-/-}\) mice and CD8\(^{+}\) Tregs are known to be an important factor in the process of Tfh cell regulation, we hypothesized that STAT4 might be an essential factor in CD8\(^{+}\) Treg differentiation. To test this hypothesis, we examined the cellularity of spleens isolated from Stat4\(^{-/-}\)Ldlr\(^{-/-}\) and Ldlr\(^{-/-}\) mice. We detected a significant increase in the number of CD8\(^{+}\)CXCR5\(^{-}\)CD275\(^{-}\)CD122\(^{+}\) T cells in Stat4\(^{-/-}\)Ldlr\(^{-/-}\) mice (p<0.05; Fig. 3B), despite a reduction in total CD8\(^{+}\) T cells in Stat4\(^{-/-}\)Ldlr\(^{-/-}\) mice (Fig. 3C). Interestingly, there was no significant difference in Tfh or CD8\(^{+}\) Treg content between spleens of Stat4\(^{-/-}\) and C57BL/6 mice (data not shown). Thus, our results suggest that STAT4 might regulate components of the humoral response in atherogenesis via the fine-tuning of a CD8\(^{+}\) Treg/Tfh/B cell axis.

It is well-known that STAT4 is a transcription factor involved in the differentiation of Th1 cells\(^{6}\) and negative regulation of CD4\(^{+}\) Treg generation\(^{8,27}\). Here we hypothesized that STAT4 might be also important as a negative regulator for the differentiation of CD8\(^{+}\) Tregs upon Treg stimulating conditions in vitro. To test this concept, we isolated CD8\(^{+}\) splenic T cells from both Stat4\(^{-/-}\)Ldlr\(^{-/-}\) and Ldlr\(^{-/-}\) mice, then cultured them with plate bound anti-CD3 in complete RPMI media supplemented with soluble anti-CD28, rhIL-2, and rhTGF\(\beta\) for 3 days as described\(^{23}\). We detected an increased percentage of STAT4-deficient CD8\(^{+}\) Tregs compared with STAT4-sufficient CD8\(^{+}\) Tregs in analyzed cell cultures (Fig. 3D) suggesting a unique cell intrinsic role of STAT4 in regulating CD8\(^{+}\) Treg development.

**Increased content of CD8\(^{+}\) Tregs but reduced number of Tfh cells in the aortas of Stat4\(^{-/-}\)Ldlr\(^{-/-}\) mice**

To further clarify the potential mechanism through which STAT4-deficiency reduces plaque formation, we examined the aortic immune composition with a specific focus on CD8\(^{+}\) Tregs, Tfh cells and M\(\Phi\)s. STAT4-deficiency was associated with an increase in aortic CD8\(^{+}\) Tregs (CD8\(^{+}\)CXCR5\(^{-}\)CD275\(^{-}\)CD122\(^{+}\) T cells) in 16 week DDC fed Stat4\(^{-/-}\)Ldlr\(^{-/-}\) mice in
comparison with $\textit{Ldlr}^{-/-}$ controls (Fig. 4A). Furthermore, STAT4-deficiency led to decreased number of Tfh cells within the aorta of $\textit{Stat4}^{-/-}\textit{Ldlr}^{-/-}$ versus $\textit{Ldlr}^{-/-}$ mice (Fig. 4B). These results indicated a potential STAT4-dependent fine-tuning of the balance of $\text{CD8}^+$ Treg and Tfh cells in the aortic wall during atherogenesis.

$\text{M}\Phi$ numbers strongly correlate with the severity of arterial inflammation and atherogenesis in mice and humans. Therefore, we sought to determine whether the presence of $\text{M}\Phi$s was also affected by STAT4-deficiency in $\textit{Stat4}^{-/-}\textit{Ldlr}^{-/-}$ aortas. We detected a significant reduction in the percentage of CD11b$^+$F4/80$^+$ cells in the aorta of $\textit{Stat4}^{-/-}\textit{Ldlr}^{-/-}$ versus $\textit{Ldlr}^{-/-}$ mice (p<0.01, Fig. 4C), with a trending decrease in number of $\text{M}\Phi$s (CD11b$^+$F4/80$^+$) in $\textit{Stat4}^{-/-}\textit{Ldlr}^{-/-}$ aortas (Fig. 4C). Altogether, STAT4 deficiency reduces the numbers of Tfh cells and $\text{M}\Phi$s within the aortic wall of $\textit{Stat4}^{-/-}\textit{Ldlr}^{-/-}$ mice, but supports the presence of $\text{CD8}^+$ Tregs within the aorta.

**Reduced number of activated splenic $\text{M}\Phi$s in $\textit{Stat4}^{-/-}\textit{Ldlr}^{-/-}$ mice**

The initiation and progression of atherosclerosis is accompanied by a complex immune response and $\text{M}\Phi$s play a key role in the modulation of arterial and systemic inflammatory processes. Here we sought to evaluate $\text{M}\Phi$ content and activation status in IR-$\textit{Ldlr}^{-/-}$ mice model and examine how $\text{M}\Phi$s might affect the $\text{CD8}^+$ Treg and Tfh cell differentiation. We detected a similar reduction in percent (data not shown), number of CD11b$^+$CD68$^+$Ly6C$^+$ (Fig. 5A), and CD11b$^+$CD68$^+$MHC-II$^+$ pro-inflammatory $\text{M}\Phi$s (Fig. 5B) in spleens of $\textit{Stat4}^{-/-}\textit{Ldlr}^{-/-}$ in comparison with $\textit{Ldlr}^{-/-}$ mice. To further investigate the potential impact of STAT4-deficiency on $\text{M}\Phi$s, we examined the gene expression of two phenotypic markers for M1 $\text{M}\Phi$ and M2 $\text{M}\Phi$ ($\text{Nos2}$ and $\text{Mrc1}$ respectively). The gene expression by $\textit{Ldlr}^{-/-}$ $\text{M}\Phi$s was set at 1, and gene expression by $\textit{Stat4}^{-/-}\textit{Ldlr}^{-/-}$ $\text{M}\Phi$s was expressed relative to $\textit{Ldlr}^{-/-}$ $\text{M}\Phi$s. As shown in Fig. 5C, we found an increase in the expression of the anti-inflammatory M2 $\text{M}\Phi$ marker $\text{Mrc1}$ in $\textit{Stat4}^{-/-}\textit{Ldlr}^{-/-}$ compared to $\textit{Ldlr}^{-/-}$ mice (p<0.01). Thus, our results suggest that STAT4-deficiency results in a reduced population of splenic $\text{M}\Phi$s and these $\text{M}\Phi$s display attenuated activation.

**STAT4-deficient macrophages inhibit Tfh but support CD8$^+$ Treg differentiation in vitro**

Tfh cell differentiation is regulated by IL-6, IL-21, IL-27, via a set of transcription factors including Bcl6, c-Maf, Batf, IRF4, and likely STAT4$^{26}$. In contrast, TGF$\beta$ and STAT5 serve as negative regulators of Tfh cell differentiation. While the major transcription factors are well characterized for Tfh cell differentiation, the transcriptional network and cytokines that drive Tfh cell differentiation in atherosclerosis are not yet studied. Here we decided to focus on STAT4-dependent $\text{M}\Phi$-derived cytokines and their ability to support Tfh cell differentiation. First, we tested whether $\text{M}\Phi$s isolated from DDC fed $\textit{Stat4}^{-/-}\textit{Ldlr}^{-/-}$ and $\textit{Ldlr}^{-/-}$ mice display a different cytokine profile upon the re-stimulation with LPS. The analysis was focused on cytokines that are known to be responsible for Tfh (IL-27, IL-6, TGF$\beta$) and CD8$^+$Treg (TGF$\beta$) differentiation$^{18,28,29}$. In Fig. 5D, the ratio represents the relative expression of analyzed genes by $\textit{Stat4}^{-/-}\textit{Ldlr}^{-/-}$ $\text{M}\Phi$s vs $\textit{Ldlr}^{-/-}$ $\text{M}\Phi$s (set up as 1). RT-PCR analysis detected no difference in the expression of $\text{I27}$ and $\text{Il6}$ between $\text{M}\Phi$s from $\textit{Stat4}^{-/-}\textit{Ldlr}^{-/-}$ and $\textit{Ldlr}^{-/-}$ mice, but revealed reduced levels of $\text{IFNg}$ expression by STAT4-
deficient MΦs (Fig. 5D). Importantly, STAT4-deficient peritoneal MΦs demonstrated an increased expression of Tgfβ (Fig. 5D).

To further explore the effects of STAT4-deficient MΦs on the differentiation of T cell subset, we analyzed how conditioned media from STAT4-deficient and STAT4-sufficient MΦs isolated from Stat4<sup>-/-</sup>Ldlr<sup>-/-</sup> and Ldlr<sup>-/-</sup> mice might affect T cell differentiation. CD4<sup>+</sup> Ldlr<sup>-/-</sup> T cells were cultured for 48 hours and then stained with hallmark transcription factors for Th1-T-bet and Treg-Foxp3. The differentiation to CD4<sup>+</sup>Tbet<sup>+</sup> or CD4<sup>+</sup>Foxp3<sup>+</sup> cells with Ldlr<sup>-/-</sup> MΦ media was set at 1, and the differentiation of CD4<sup>+</sup>Tbet<sup>+</sup> or CD4<sup>+</sup>Foxp3<sup>+</sup> cells with Stat4<sup>-/-</sup>Ldlr<sup>-/-</sup> MΦ media was expressed relative to Ldlr<sup>-/-</sup> MΦ effects. (Fig. 5E). We found that the conditioned media from Stat4<sup>-/-</sup>Ldlr<sup>-/-</sup> MΦs stimulated less differentiation into CD4<sup>+</sup>Tbet<sup>+</sup> Th1 cells, but surprisingly had no significant effect on CD4<sup>+</sup>Foxp3<sup>+</sup> Treg differentiation (Fig. 5E). Next, we analyzed the impact of conditioned media from activated Stat4<sup>-/-</sup>Ldlr<sup>-/-</sup> and Ldlr<sup>-/-</sup> MΦs on the generation of CD4<sup>+</sup>CXCR5<sup>+</sup> Tfh cells. We used the levels of Tfh cell differentiation in conditioned media from Ldlr<sup>-/-</sup> MΦs as basal levels of Tfh cell generation (set up as 1) and calculated a relative ratio of Tfh cells generated under different conditions (Fig. 5F). TGFβ is a cytokine that has been shown to inhibit differentiation of mouse Tfh<sup>15,28</sup> and support CD8<sup>+</sup>Treg differentiation.<sup>28,30</sup> Therefore, anti-TGFβ blocking Abs were used to test a role of TGFβ in Tfh cell differentiation for some experiments. Our data revealed a reduction of Tfh cell differentiation using conditioned media from LPS-stimulated Stat4<sup>-/-</sup>Ldlr<sup>-/-</sup> MΦs (Fig. 5F). This reduction in Tfh cell differentiation was TGFβ-dependent as blocking anti-TGFβ Abs restored the rate of Tfh cell generation (Fig. 5F).

Next, we tested whether the condition media from Stat4<sup>-/-</sup>Ldlr<sup>-/-</sup> MΦs could modulate differentiation of CD8<sup>+</sup> Tregs. The differentiation of CD8<sup>+</sup>Tregs in media from Ldlr<sup>-/-</sup> peritoneal MΦs isolated was set up as 1. We detected a significant increase in CD8<sup>+</sup>Treg differentiation upon co-culture of CD8<sup>+</sup> cells with Stat4<sup>-/-</sup>Ldlr<sup>-/-</sup> MΦ-derived media and this effect was reduced by the co-culture with anti-TGFβ Abs (Fig. 5G). Thus, STAT4 deficiency in MΦs results in altered cytokine profile that at least via low levels of TGFβ expression affects generation of Tfh cells and CD8<sup>+</sup> Tregs.

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Defective control of germinal center formation by STAT4-deficient CD8<sup>+</sup> Tregs in vivo

Our data demonstrate that STAT4 directly affects CD8<sup>+</sup>Treg differentiation in a cell-intrinsic manner as well as modulates differentiation of CD8<sup>+</sup>Treg indirectly, via MΦ-released cytokines. To further examine a direct impact of STAT4-deficiency on CD8<sup>+</sup>Treg functions, we tested an ability of STAT4-deficient CD8<sup>+</sup>Treg to control Tfh cell differentiation in vivo. FACS sorted STAT4-deficient (KO) or STAT4-sufficient (WT) CD8<sup>+</sup>CD122<sup>+</sup>Tregs (CD45.2<sup>+</sup>) were adoptively transferred into C57BL/6 (CD45.1<sup>+</sup>) mice followed by immunization with KLH in CFA. This is a well-established protocol that stimulates generation of Tfh and germinal center B cells in vivo.<sup>14</sup> Compared to mice receiving no cells (control/ctrl) or STAT4-sufficient (WT) CD8<sup>+</sup>CD122<sup>+</sup>Tregs, the recipients of STAT4-deficient CD8<sup>+</sup>CD122<sup>+</sup>Tregs (KO) exhibited greatly decreased percentage of CD4<sup>+</sup>CXCR5<sup>+</sup>Bcl6<sup>+</sup> Tfh cells (Fig. 6A). The transfer of Stat4<sup>-/-</sup>CD8<sup>+</sup>CD122<sup>+</sup>Treg cells also inhibited generation of B220<sup>+</sup>GL-7<sup>+</sup>Fas<sup>+</sup> germinal center B cells and KLH-specific
antibody production including IgG, IgM and IgA (Fig. 6B). These results provide a first proof of principal for a cell-intrinsic role of STAT4 in the negative regulation of CD8+ Treg functions such as a control of Tfh cell development and germinal center B cell-dependent humoral immune response in vivo.

**STAT4-deficient CD8+Tregs suppress atherosclerosis in DDC fed Ldlr−/− mice via the control of Tfh cells and humoral response**

Our data clearly indicate that STAT4-deficient CD8+ Tregs suppress the formation of germinal centers upon immunization with KLH via suppression of Tfh cell differentiation in immunized C57BL/6 recipients (Fig. 6). To further investigate the impact of CD8+ Treg–specific STAT4 deficiency in atherogenesis, we performed the adoptive transfer of sorted CD8+ Tregs from Stat4−/−Ldlr−/− or Ldlr−/− mice into 8 week-old Ldlr−/− recipients. To evaluate a basal atherosclerotic plaque formation, we used 8 week-old Ldlr−/− recipients with no injection of CD8+ Tregs (PBS-control). After 11 weeks of DDC feeding, all recipient mice were analyzed for plaque burden in aortas. In average, 11 week DDC fed Ldlr−/− mice developed 13.7±0.5 % of plaque throughout the aorta (n=6). In agreement with the previous report by Clement et al., the adoptive transfer of WT CD8+CD122+ Treg reduced plaque formation in comparison with PBS-control Ldlr−/− recipients, indicating a suppressive, atheroprotective role of CD8+ Tregs in atherosclerosis (13.7±0.5 % vs 10.1±1.2, p<0.05). Importantly, the adoptive transfer of Stat4−/−Ldlr−/− (KO) CD8+CD122+ Tregs resulted in further reduction in plaque burden in Ldlr−/− recipients in comparison with the plaque development in Ldlr−/− mice that received Ldlr−/− (WT) CD8+CD122+ Tregs (3.6±1.2 vs 10.1±1.2, respectively, p<0.05; Fig. 7A). Transferred Stat4−/−Ldlr−/− CD8+ Tregs also significantly diminished the percentage of CD4+CXCR5+Bcl6+ Tfh cells in the spleens and para-aortic lymph nodes (para-aortic LN) of the Ldlr−/− recipients in comparison with Ldlr−/− mice that received Ldlr−/− CD8+ Tregs (Fig. 7B). These results suggest that STAT4-deficient CD8+ Tregs not only impact the local LN but also affect a systemic immune response in the Ldlr−/− recipients. The transfer of Stat4−/−Ldlr−/− CD8+ Tregs also suppressed the generation of B220+GL-7+FAS+ germinal center B cells in para-aortic LN of the Ldlr−/− recipients (Fig. 7B–C). In parallel, attenuated levels of circulating IgG1, IgG3, and IgA antibodies were detected in the plasma of Ldlr−/− mice that received Stat4−/−Ldlr−/− CD8+ Treg vs Ldlr−/− CD8+ Treg recipients (Fig. 7D). Overall, these results clearly demonstrate an important cell-intrinsic role of STAT4 in the regulation of CD8+ Treg functions and provide strong evidence for the importance of STAT-4-dependent role of CD8+ Tregs in the regulation of Tfh and germinal center B cell development in atherosclerosis.

**Discussion**

STAT4 is a major transcription factor regulating adaptive immune responses but also plays a detrimental role in several autoimmune diseases. In this study we investigate a role of STAT4 in a complex model of atherogenesis under conditions of insulin resistance. In this report, we demonstrate that STAT4 deficiency results in reduced plaque formation at early and advanced stages of atherosclerosis caused by a cholesterol-containing diabetogenic diet resulting in concomitant insulin resistance. A key novelty of this study is that we show unexpected effects of STAT4 deficiency on the modulation of CD8+ Treg and Tfh cell development.
content in atherosclerosis-prone conditions. STAT4-deficiency supports CD8\(^+\) Treg differentiation \textit{in vitro} and CD8\(^+\) Treg functions \textit{in vivo} suggesting CD8\(^+\) cell-intrinsic effects of STAT4. Additionally, STAT4 deficiency not only affects T cell fate, but also directly diminishes pro-inflammatory M\(\Phi\) phenotype that in turn supports CD8\(^+\) Treg differentiation and a partial abolishment of Tfh cell development via a TGF\(\beta\)-dependent mechanism. Altogether, our data uncover a pro-inflammatory role of STAT4 in IR-accelerated atherosclerosis and highlight a new exciting role of STAT4 in the regulation of the Tfh/CD8\(^+\) Treg axis.

Numerous reports indicate the association between insulin resistance, hyperlipidemia, glucose intolerance, hypertension, obesity, and cardiovascular disease\(^{31,32}\). One of the limitations in this area of investigation is a restricted number of mouse models that can be used for studying pro-atherogenic effects of insulin resistance or type 2 diabetes\(^4\). To overcome this limitation, Schreyer and colleagues established a combined model of IR and atherosclerosis, and later Subramanian et al. further characterized DDC fed \textit{Ldlr}\(^{-/-}\) mice with the specific focus on atherosclerosis and adipose tissue inflammation\(^5,24\). In this study, we used this model to study effects of STAT4 deficiency on IR-accelerated atherogenesis. Our results demonstrate that STAT4 is a powerful pro-atherogenic transcription factor in the conditions of insulin resistance as Stat4\(^{-/-}\) \textit{Ldlr}\(^{-/-}\) mice show significantly attenuated atherosclerotic plaque burden upon 16 or 24 weeks DDC feeding.

It has been established that STAT4 as well as T-bet are necessary for full differentiation of Th1 cells\(^7,12,33\). However, recent studies have shown that STAT4 is also involved in the regulation, differentiation, and activation of several cell types including: NK cells, monocytes, M\(\Phi\)s, DCs, and Th cell lineages\(^9,34,35\). Here, in the model of IR-accelerated atherosclerosis we detected a reduction in the number and activation of splenic M\(\Phi\)s and CD3\(^+\) T cells as well as the diminished number of M\(\Phi\)s, and Tfh cells within the aorta. In contrast, the CD8\(^+\) Tregs population was elevated in Stat4\(^{-/-}\) \textit{Ldlr}\(^{-/-}\) mice in comparison with the \textit{Ldlr}\(^{-/-}\) controls. Thus, our data indicates that STAT4 regulates the systemic pro-inflammatory response via the modulation of various immune cells in atherosclerosis.

CD8\(^+\) Tregs that are specific for the Qa-1 molecule, an unconventional MHC class I molecule in mice, control autoreactive CD4\(^+\) T cells and Qa-1\(^+\) Tfh cells\(^20\). Numerous body of evidence suggests an important role of CD8\(^+\) Tregs in human autoimmune diseases\(^36\) and a recent study also highlights the role of CD8\(^+\) Tregs and the Tfh/germinal center B cell axis in atherosclerosis\(^13\). In this study, we underscore a critical role of STAT4 in the regulation of the CD8\(^+\)Treg/Tfh cell axis in atherogenesis. We demonstrate that STAT4 plays a critical role in the formation and suppressive functions of CD8\(^+\)Tregs during atherogenesis.

To date, a transcriptional network that directs the differentiation of CD8\(^+\) Tregs has not been identified. There is evidence that Helios and STAT5 are required for CD8\(^+\) Treg differentiation and survival\(^26\). Our results indicate that STAT4-deficiency leads to an increase in splenic and aortic CD8\(^+\) Tregs, accompanying by a reduction in Tfh cells and plasma B cells in DDC fed Stat4\(^{-/-}\) \textit{Ldlr}\(^{-/-}\) mice. Furthermore, we present data suggesting that STAT4 can support CD8\(^+\) Treg differentiation from CD8\(^+\) cells in cultures under \textit{in vitro} Treg polarizing conditions suggesting cell-intrinsic effects of STAT4 in CD8\(^+\) cell

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phenotype. It is well established that STAT4 suppresses Foxp3 expression and therefore CD4+ Treg differentiation in vitro. The expression of Foxp3 in CD8+ Tregs is up to much debate. Future studies will be necessary to dissect STAT4-dependent molecular signaling pathways that drive CD8+ Treg differentiation. Importantly, the effects of STAT4 deficiency are not only restricted to the regulation of CD8+ Treg differentiation as we demonstrate that STAT4 deficiency also affects CD8+ Treg functions. Adoptively transferred STAT4-deficient CD8+ Tregs significantly suppress the development of Tfh cells, germinal center B cells, and a subsequent synthesis of immunoglobulins in KLH-immunized BL/6 recipients. These results provide a first proof-of-principle for a cell-intrinsic role of STAT4 in CD8+ Treg functions. In this report, we also demonstrate that STAT4-deficient CD8+ Tregs effectively suppress differentiation of Tfh cells and germinal B cell formation upon atherogenesis. Importantly, the adoptive transfer of STAT4-deficient CD8+ Tregs considerably reduced atherosclerotic plaque formation indicating the functional importance of STAT4 deletion in CD8+ Tregs. Altogether we demonstrate that STAT4 is a transcription factor that is crucial for the differentiation and functions of CD8+ Tregs in vivo under inflammatory conditions.

Tfh cell differentiation is regulated by IL-6 and IL-21 via Bcl6 that is required for Tfh cell commitment and STAT3 is likely also involved in Tfh cell fate decision. While the major transcription factors are well characterized for Tfh cell differentiation, the transcriptional network and cytokines that drive Tfh cell differentiation in atherosclerosis is an area under active investigation. There have been studies that indicate the inverse roles of both IL-27 and TGFβ in the generation and function of mouse Tfh cells. IL-27 is a cytokine that enhances the functionality of Tfh cells within the murine model of lupus. In contrast, Tfh cells display reduced differentiation in the presence of TGFβ. Interestingly, mouse studies have indicated a strong correlation between the activation of the IL-12/STAT4 pathway and the reduction in TGFβ in chronic autoimmune diseases such as rheumatoid arthritis.

MΦs are the central cells in atherosclerosis and regulate the development of the disease in multiple ways such as modulation of cholesterol metabolism and production of immunomodulatory chemokines and cytokines. As IL-27 and TGFβ expression is important for Tfh cell differentiation, we decided to further test a role of MΦ-derived cytokines and examine the levels of IL-27 and TGFβ expression in activated MΦs isolated from insulin resistant Stat4−/−Ldlr−/− mice. We observed no difference in the expression level of Il27 upon a STAT4-deficiency; however, we did detect an increase in the expression of Tgfβ in STAT4-deficient MΦs. Next, to uncover potential impacts of STAT4-dependent MΦ functions on the regulation of T cell polarization, we simulated isolated Ldlr−/− CD4+ T cells with the media collected from STAT4-sufficient and STAT4-deficient LPS-activated peritoneal MΦs. Ldlr−/− CD4+ T cells plated in the media collected from STAT4-deficient LPS-activated peritoneal MΦ had a reduced ability to differentiate into Tfh cells. Interestingly, several studies have also revealed that TGFβ is necessary for the development of CD8+ Tregs. In line with this notion, we detected preferential CD8+ Treg generation using STAT4-deficient MΦ-released media. These results suggest that the loss of MΦ-specific STAT4 alters MΦ activation, increases TGFβ production, and fine-tunes the content of Tfh and CD8+ Tregs. Thus, STAT4 might affect the CD8+ Tregs/Tfh axis in two ways: directly via the regulation of CD8+ Treg differentiation/functions and Tfh cell suppression.
and indirectly via STAT4-dependent MΦ-dependent effects on the CD8+ Treg and Tfh cell differentiation.

In summary, STAT4 participates in atherogenesis via the support of pro-inflammatory MΦ activities, regulation of CD8+ Treg/Tfh cell axis, and the modulation of the local immune response in the aortic wall under conditions of IR and atherosclerosis. Notably, our data uncovered a key role of STAT4 in the negative regulation of CD8+ Treg differentiation and suppressive functions in vivo. The obtained results suggest that modulation of STAT4 expression could provide a novel therapeutic approach to reducing accelerated atherosclerosis associated with IR and diabetes.

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Reference List


Figure 1. STAT4-deficiency attenuates atherosclerosis and improves metabolic parameters in Stat4<sup>−/−</sup>Ldlr<sup>−/−</sup> mice fed a diabetogenic diet with added cholesterol (DDC).

(A) Glucose tolerance test (GTT) and (B) insulin tolerance tests (ITT) from Stat4<sup>−/−</sup>Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup> male mice fed Chow Diet (CD) or DDC diets for 15 weeks (n=8–11 mice per genotype). AUC: the area under the curve. (C–E) Representative en face Oil Red O staining of aortas from (C) 16 weeks (E) 24 weeks CD and DDC diet fed male Stat4<sup>−/−</sup>Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup> mice. Lesion size (% of whole aorta) is shown. Aortic root from (D) 16 week DDC diet fed male Stat4<sup>−/−</sup>Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup> mice were stained with MOVAT staining and plaques areas were measured. The data depicts the mean±SEM. Each symbol represents 1 animal; horizontal bars represent means. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.001.
Figure 2. Stat4-deficiency affects Tfh and plasma B cell content in Stat4−/−Ldlr−/− mice fed DDC diet

16 week DDC fed Stat4−/−Ldlr−/− and Ldlr−/− splenic cell suspensions were stained with anti-CD3, CD4, CD44, CD69, CXCR5, CD19, CD21, and CD138 Abs and analyzed by flow cytometry. (A) Decreased number of splenic CD4+ T cells and (B) CD3+CD44+ and CD3+CD69+ T cells in the spleens of Stat4−/−Ldlr−/− (black bars) compared with Ldlr−/− (grey bars) mice (n=8–12 per genotype). (C) The total number of Tfh cells was decreased in the spleens of Stat4−/−Ldlr−/−(black bars) compared with Ldlr−/− (grey bars) mice (n=6–7 per genotype). (D) Representative flow cytometry plots for CD4+CXCR5+ Tfh cells. (E) The total number of CD19+CXCR5−CD138+CD21− plasma cells was decreased in the spleens of Stat4−/−Ldlr−/− (black bars) compared with Ldlr−/− (grey bars) mice. (F) Representative FACS plots from Stat4−/−Ldlr−/− and Ldlr−/− spleens (n=7–8 per genotype). *p < 0.05, **p < 0.01, ****p<0.0001.
Figure 3. STAT4 deficiency increases CD8+ Treg numbers in the spleen of Stat4−/−Ldlr−/− mice and supports CD8+ Treg differentiation in vitro
16 week DDC fed Stat4−/−Ldlr−/− and Ldlr−/− splenic cell suspensions were stained with anti-CD3, CD8, CXCR5, CD275, CD122 Abs and analyzed by flow cytometry. (A) Flow cytometry gating scheme for CD8+ Tregs. (B) Increased number of CD8+ Tregs despite decreased content of total splenic CD8+ T cells (C) in Stat4−/−Ldlr−/− (black bars) compared with Ldlr−/− (grey bars) mice (n=7–8 mice per genotype). Representative histograms are shown. (D) In vitro differentiation of Stat4-deficient and Stat4-sufficient CD8+ Tregs with plate bound ant-CD3 (1ug/mL) in complete RPMI media supplemented with soluble anti-CD28 (1ug/mL), rhIL-2 (100 U/mL), and rhTGFβ (2ng/mL) for 3 days (n=6, 3 independent experiments). Representative histograms in the CD8+CXCR5+CD275+ gates are shown. *p < 0.05, **p < 0.01
Figure 4. Increased number of CD8+ Tregs and a concomitant reduction of Tfh cells and macrophages within the aortas of Stat4−/−Ldlr−/− mice

(A) Elevated number of CD8+ Tregs within the aortas of Stat4−/−Ldlr−/− (black bars) compared with Ldlr−/− (grey bar) mice (3 pooled aortas per genotype, n=6 in 3 independent experiments). Representative histograms of aortic CD8+ Tregs (CD8+CXCR5+CD275+CD122+) from Stat4−/−Ldlr−/− and Ldlr−/− mice are shown. Gates are based on isotype control staining. (B) Reduced number of Th cells within the aortas of Stat4−/−Ldlr−/− (black bars) compared with Ldlr−/− (grey bar) mice (n=8–12 per genotype). Representative FACS plots and histograms for aortic Tfh cells from Stat4−/−Ldlr−/− and Ldlr−/− mice. (C) Content of CD11b+F4/80+ MΦs from the aortas of Stat4−/−Ldlr−/− (black bars) versus Ldlr−/− (grey bars) mice (n=8–9 per genotype). **p < 0.01, ****p<0.0001.
Figure 5. STAT4 deficiency induces anti-inflammatory MΦ phenotype that supports development of CD8\(^+\) Tregs, but not Tfh cells via a TGF\(\beta\)-dependent mechanism

(A–B) Splenic cell suspension from 16 week DDC fed Stat4\(^{-/-}\)Ldlr\(^{-/-}\) (black bars) and Ldlr\(^{-/-}\) (grey bars) mice were stained with anti-CD45, CD11b, CD68, I-A\(^b\), and Ly6C Abs and analyzed by flow cytometry (n=5–6 per genotype). (C) RT-PCR expression of Nos2 and Mrc1 in MΦs isolated from Stat4\(^{-/-}\)Ldlr\(^{-/-}\) (black bars) and Ldlr\(^{-/-}\) (grey bars) mice (n=4 mice). Results show mean±SE as the ratio of Stat4\(^{-/-}\)Ldlr\(^{-/-}\) (black bar) to Ldlr\(^{-/-}\) (grey bar) MΦs. (D) Peritoneal MΦs from 16 week DDC fed Stat4\(^{-/-}\)Ldlr\(^{-/-}\) (black bars) and Ldlr\(^{-/-}\) (grey bars) were collected, stimulated with LPS, and analyzed by RT-PCR. The ratio from Stat4\(^{-/-}\)Ldlr\(^{-/-}\) and Ldlr\(^{-/-}\) MΦs is shown (n=5–6 per genotype). (E–G) Stat4\(^{-/-}\)Ldlr\(^{-/-}\) and Ldlr\(^{-/-}\) peritoneal MΦs were isolated and activated with LPS (500ng/mL) in culture for 48 hours. Isolated CD4\(^+\) cells were cultered with plate bound anti-CD3, soluble anti-CD28, and the conditioned media (CM) for 72 hrs. Differentiated cells were stained for Foxp3, Tbet, CXCR5, CD8, CD4, CD122, and analyzed by flow cytometry. (E) Differentiation of Th1 cells (CD4\(^+\)Tbet\(^+\)) and Tregs (CD4\(^+\)Foxp3\(^+\)) with MΦ-derived conditioned media. Results show mean±SE as the ratio of Stat4\(^{-/-}\)Ldlr\(^{-/-}\) to Ldlr\(^{-/-}\) of Th1 or Tregs (n=6–8 mice, 4 independent experiments). (F–G) Differentiation of CD8\(^+\) Tregs and Tfh cells with MΦ-derived CM without and with the addition of 2 μg/mL of anti-TGF\(\beta\) Abs. Results show mean±SE as the ratio of Stat4\(^{-/-}\)Ldlr\(^{-/-}\) to Ldlr\(^{-/-}\) of Tfh or CD8\(^+\) Tregs, n=6, 2 independent experiments). *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001.
Figure 6. Defective control of germinal center formation by STAT4-deficient CD8+Tregs

STAT4-sufficient or STAT4-deficient sorter CD8+CD122+ (CD45.2+) Tregs were adoptively transferred into C57BL/6 (CD45.1+) recipient mice (n=4/per group). A group of C57BL/6 mice that did not receive T cells was used as a control. All mice were immunized subcutaneously with 1000 μg KLH emulsified in CFA. Seven days after immunization, cell suspensions from draining lymph nodes of the recipients were stained with anti-Bcl6, CD4, CXCR5, GL-7, Fas, B220, and Tfh cells and germinal center B cells were analyzed. (A) Representative FACS plots from recipients that received WT CD8+ Tregs or STAT4-deficient CD8+ Tregs or no cells (ctrl). Numbers in the boxes represent the percentages. (B) The sera from the recipients were subject to a 3-fold serial dilution, and the concentrations of KLH-specific IgG, IgM, and IgA were analyzed by ELISA. *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 7. Adoptively transferred STAT4 deficient CD8+Tregs control Tfh and germinal B cell development in Ldlr−/− recipient mice

Stat4−/−Ldlr−/− and Ldlr−/− sorted CD8+CD122+ Tregs were adoptively transferred into Ldlr−/− recipient mice (n=5/per group). After 11 weeks of DDC feeding, aortas were analyzed for plaque burden and cell suspensions from para-aortic and peripheral LN were stained with anti-Bcl6, CD4, CXCR5, GL-7, FAS, B220, and Tfh cells and germinal center B cells were analyzed. (A) Representative en face Oil Red O staining of aortas from DDC fed Ldlr−/− recipients that received either Stat4−/−Ldlr−/− (KO) or Ldlr−/− (WT) sorted CD8+CD122+ Tregs. Lesion size (% of whole aorta) is shown. Each symbol represents 1 animal; horizontal bars represent means. (B) Representative FACS plots from recipients that received either Stat4−/−Ldlr−/− (KO) CD8+ Tregs or Ldlr−/− (KO) CD8+ Tregs. Numbers in the boxes represent the percentages. (C) Decreased percentage of Tfh cells and FAS+GL7+B-220+ cells in para-aortic LN and Tfh cells in the spleen of Ldlr−/− recipients that received Stat4−/−Ldlr−/− CD8+ Tregs (blue bars) vs Ldlr−/− mice that received Ldlr−/− CD8+ Tregs (red bars) (n=4–5 per genotype). (D) Reduced levels of IgG1, IgG3, and IgA in the plasma of Ldlr−/− mice received Stat4−/−Ldlr−/− CD8+ Tregs (blue bars) vs Ldlr−/− mice that received Ldlr−/− CD8+ Tregs (red bars). *p < 0.05, **p < 0.01, ***p < 0.001.