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# STAT4 deficiency reduces the development of atherosclerosis in mice

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ABSTRACT

Atherosclerosis is a chronic inflammatory process that leads to plaque formation in large and medium sized vessels. T helper 1 (Th1) cells constitute the majority of plaque infiltrating pro-atherogenic T cells and are induced via IFNγ-dependent activation of T-box (Tbet) and/or IL-12-dependent activation of signal transducer and activator of transcription 4 (STAT4). We thus aimed to define a role for STAT4 in atherosclerosis. STAT4-deficiency resulted in a ~71% reduction ($p < 0.001$) in plaque burden in *Stat4*$^{-/-}$*Apoe*$^{-/-}$ vs *Apoe*$^{-/-}$ mice fed chow diet and significantly attenuated atherosclerosis (~31%, $p < 0.01$) in western diet fed *Stat4*$^{-/-}$*Apoe*$^{-/-}$ mice. Surprisingly, reduced atherogenesis in *Stat4*$^{-/-}$*Apoe*$^{-/-}$ mice was not due to attenuated IFNγ production *in vivo* by Th1 cells, suggesting an at least partially IFNγ-independent pro-atherogenic role of STAT4. STAT4 is expressed in T cells, but also detected in macrophages (MΦs). *Stat4*$^{-/-}$*Apoe*$^{-/-}$ *in vitro* differentiated M1 or M2 MΦs had reduced cytokine production compare to *Apoe*$^{-/-}$ M1 and M2 MΦs that was accompanied by reduced induction of CD69, I-A$^{b}$, and CD86 in response to LPS stimulation. *Stat4*$^{-/-}$*Apoe*$^{-/-}$ MΦs expressed attenuated levels of CCR2 and demonstrated reduced migration toward CCL2 in a transwell assay. Importantly, the percentage of aortic CD11b$^{+}$F4/80$^{+}$Ly6C$^{hi}$ MΦs was reduced in *Stat4*$^{-/-}$*Apoe*$^{-/-}$ vs *Apoe*$^{-/-}$ mice. Thus, this study identifies for the first time a pro-atherogenic role of STAT4 that is at least partially independent of Th1 cell-derived IFNγ, and primarily involving the modulation of MΦ responses.



## 1. Introduction

Atherosclerosis is a disease of large and medium sized vessels that is accompanied by chronic inflammation in the arterial wall due to the involvement of the innate and adaptive immune responses [1,2]. While macrophages (MΦs) are known to be essential and the most prominent and abundant leukocyte subset within the atherosclerotic aorta [1,3], several subpopulations of T cells are also detected within the atherosclerotic plaques, including T regulatory (Treg), T helper 1 (Th1), Th2, and Th17 cells, with IFNγ-producing Th1 cells being the most abundant [4]. Th1 cells release IFNγ causing the activation of MΦs and DCs, which generates a feedback loop leading to the Th1-driven pathogenesis and lesion amplification [4,5].

The development and functions of Th1 cells are dependent on several transcription factors including the signal transducer and activator of transcription 4 (STAT4) and a member of the T box family of transcription factors, T-bet (*Tbet/Tbx21*) [6–8]. It was suggested that T-bet serves as a master regulator for the development of Th1 cells; however, STAT4 is also required for the complete differentiation of Th1 cells [9]. Additional evidence indicates that T-bet and STAT4 might not be serving in a linear pathway and each factor can play a unique role in programming chromatin architecture for Th1 gene expression [9]. Significant reduction of Th1 cells via the deletion of Tbx21 attenuates lesion plaque formation in Tbet-deficient *Ldlr*$^{-/-}$ mice indicating a pro-atherogenic role of T-bet and Tbet-dependent Th1 cells in atherosclerosis [10]. While the

*Non-standard Abbreviations and Acronyms:* Apoe, Apolipoprotein E; WD, Western Diet; MΦ, Macrophages; PLN, Peripheral Lymph Node.

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role of T-bet in atherosclerosis is well established, the potential impact of STAT4 on atherogenesis is unclear.

IL-12 is one of the key cytokines that induces Th1 cell differentiation upon acute and chronic inflammation. Importantly, functional blockade of endogenous IL-12 by vaccination resulted in attenuated atherosclerosis accompanied by improved plaque stability [11], indicating an important role of IL-12-dependent pathways during atherogenesis. IL-12 is a major factor for STAT4 phosphorylation in T cells resulting in their increased activation as well as development of fully functional Th1 cells and Type I IFNs are responsible for the activation of STAT4 in NK cells [7,12]. IL-23 is a cytokine that shares the IL-12p40 receptor subunit and activates the same JAK-STAT signaling molecules, but only weakly activates STAT4 [13]. Interestingly, STAT4 also limits Treg development [14], and therefore is involved in the regulation of the delicate balance between Th1 and Treg cells. While, much of work on STAT4 has been performed in T cells, STAT4 is also expressed in the myeloid lineage, mainly in activated monocytes, MΦs, and DCs [15].

In line with the involvement of STAT4 in the regulation of activity of leukocyte subsets, multiple reports demonstrated an important role of STAT4 in several pathological conditions. Mice that are deficient in *Stat4* have decreased IFNγ production and are resistant to T cell-related autoimmune diseases such as experimental autoimmune encephalomyelitis [16], type I diabetes [17], and are susceptible to certain intracellular pathogens [7]. Notably, recent studies also demonstrated an important role of the IL-12/STAT4 axis in the regulation of vascular injury response [18]. STAT4-deficiency also decreases neointima formation under conditions of insulin resistance in obese Zucker rats [19].

Based on the regulatory role of STAT4 in Th cell differentiation and myeloid cell activation/functions, we hypothesized that STAT4 would play a role in atherosclerosis. Here, we demonstrate that STAT4-deficiency results in the attenuated MΦ activation, diminished aortic MΦ content, and reduced atherosclerosis in *Stat4*-deficient Apolipoprotein E-deficient (*Stat4*$^{-/-}$*Apoe*$^{-/-}$) mice. Interestingly, the effects of STAT4 are seen despite continued IFNγ production by Th1 cells.

## 2. Materials and methods

### 2.1. Animals

*Stat4*$^{-/-}$ mice [20] on the C57BL/6 background were cross-bred with *Apoe*$^{-/-}$ mice to generate *Stat4*$^{-/-}$*Apoe*$^{-/-}$ mice. Seven week-old *Stat4*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ were fed a chow diet for 27 weeks or Western diet (21% fat and 0.15% cholesterol, Harlan Teklad, Harlan Laboratories, Indianapolis, IN) for 12 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.

### 2.2. En face

Aortas were harvested and stained for atherosclerotic lesions using Oil Red O as previously described [21]. Analysis of atherosclerotic area was determined by ImageJ software. Hearts were harvested then fixed with 4% PFA via cardiac puncture. Immunohistochemistry was conducted on sequential 5 μm aortic root sections cut from the point of appearance of the aortic valve leaflets [21]. Six sequential 5 μm-thick aortic root sections >150 μm were collected and analyzed by Russell modified-Movat staining as previously described [21].

### 2.3. Measurement of plasma lipids

Total plasma cholesterol levels, plasma HDL and LDL were determined using Wako colorimetric kits and protocol (Wako Diagnostics, Wako Chemicals USA Inc., Richmond, VA).

### 2.4. Quantitative real time PCR

Total RNA was extracted from splenic cells and MΦs using Trizol® reagent (Invitrogen™, Life Technologies, Grand Island, NY). DNase I treatment using RNeasy kits was used to remove contaminating genomic DNA (Qiagen, Germantown, MD). Splenic RNA: Approximately 1 μg of total RNA was reverse transcribed to cDNA by synthesis reactions containing random hexamers, 10 mM dNTPs, Moloney murine leukemia virus reverse transcriptase (MMLV), 0.1 M DTT, and 5x1$^{st}$ strand buffer (Sigma–Aldrich, St. Louis, MO). Real time PCR was performed using Taqman probes from Applied Biosystems (Carlsbad, CA), 10 mM dNTPs, 10x PCR buffer without $MgCl_2$, $MgCl_2$, and Jumpstart Taq polymerase (Sigma–Aldrich, St. Louis, MO) for *Ifnγ* and *Il17a* for splenic cells, and *iNos, Mrc1, Arg1*, and *Retnla* for MΦs. 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 *Actb* or *18S*.

### 2.5. Flow cytometry

Single cell suspensions from the aorta were prepared as previously described [21]. Briefly, mice were anesthetized using $CO_2$, blood was collected via cardiac puncture and erythrocytes were lysed using ACK lysing buffer (8.29 mg/ml $NH_4CL$, 1 mg/ml $KHCO_3$, 0.372 mg/ml EDTA, all from Sigma–Aldrich). 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 h 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 [21]. Aortas, spleens, and peripheral lymph node (PLN), were delicately rubbed in a 70 μm cell sieve (Corning Incorporated Life Sciences, Tewksbury, MA). Cell numbers were determined using trypan blue (MP Biomedicals, LLC, Solon, OH) and the hemocytometer. For intracellular cytokine staining, splenic and aortic cell suspensions were cultured for 5 h in RPMI 1640 supplemented with 10% FBS, 1% Penicillin/Streptomycin, 10 ng/ml PMA, 500 ng/ml calcium ionophore and GolgiStop (BD Biosciences, San Jose, CA). Intracellular staining for IFNγ and IL-17A, as well as CD68 was performed according to the Fix&Perm® cell permeabilization protocol (BD Biosciences, San Jose, CA). The Cytek DXP 8 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.

### 2.6. Bone marrow-derived macrophages (BMDM)

BMDM were isolated according to Zhang et al. [22]. Briefly, femurs were cleaned of tissue then cut at joints exposing the bone marrow cavity. Cold PBS was flushed through the cavity. Cells were plated in DMEM/F12 media with 10 ng/mL GM-CSF for 7 days. On day 3, the media was then re-supplemented with 10 ng/mL GM-CSF.