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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.

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Disclosures. None

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Keywords

Atherosclerosis; Inflammation; Leucocytes; Transcription factors

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 proatherogenic role of T-bet and Tbet-dependent Th1 cells in atherosclerosis[10]. While the 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

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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.

Materials and Methods

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 Taklad, 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.

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 m-thick aortic root sections >150μm were collected and analyzed by Russell modified-Movat staining as previously described[21].

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).

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 5×1st strand buffer (Sigma-Aldrich, St. Louis, MO). Real time PCR was performed using Taqman probes from Applied Biosystems (Carlsbad, CA), 10 mM dNTPs, 10× 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™

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Real-Time System C1000™ Thermal Cycler detection system (Bio-Rad laboratories). The results were normalized to housekeeping gene *Actb* or *18S*.

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 (8.29mg/ml NH_4CL, 1mg/ml $KHCO_3$, 0.372mg/ml EDTA, all from Sigma-Aldritch). 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[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 hours 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.

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 10ng/mL GM-CSF for 7 days. On day 3, the media was then re-supplemented with 10ng/mL GM-CSF.

Macrophage and BMDM polarization

Briefly, cells 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 cells. Lymphocytes were removed by washing with complete media. To prime classical M1s, IFNγ (150 U/mL) was added to complete media and incubated for 6 hours. Then M1s were stimulated by adding LPS (100 ng/mL) for 16 hours. M2 were primed by adding IL-4 (20 U/mL and IL-13 (15 U/mL) in complete media to cells and incubated overnight. Then supernatants for both M1 and M2 were collected and cytokines were detected using IL-10, IL-12p70, IFNγ, TNFα, CCL2, and CXCL10 Flow Cytomix assays (eBioscience, San Diego, CA). Data are presented as a fold change compared to the control *Apoe*$^{-/-}$ (1 fold). *Stat4*$^{-/-}$ and wild type BMDM were activated with Heat-killed methicillin resistant *Staphylococcus aureus* (HK-MRSA, generously provided by Dr.Serezani, Indiana

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University) (10 bacteria: 1 macrophage) for 24 hours, then incubated with IL-12 (5 ng/mL) for 6 hours and stimulated with LPS (100 ng/mL) for 18 hours. Cytokine concentrations were determined by multiplex assay for IL-10, IFNγ, TNFα, CCL2 and CXCL10.

LPS stimulation

Splenic cell suspensions were cultured in complete RPMI-1640 supplemented media with 10% FBS, 1% Penicillin/Streptomycin, 1% Glutamax, 1% Hepes, 0.5% NEAA, 0.5% Sodium pyruvate, 50 μM BME, and LPS (5 μg/mL) for 16 hours. Cells were stained with anti-CD86, CD11b, and MHC-II (I-A^b) Abs and analyzed by flow cytometry. Data are presented as a fold change (percentage of positive cells) compared to the control *Apoe*$^{-/-}$ (1 fold).

Th1/Treg polarization

Naive splenic $CD4^+$ cells from *Stat4*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice were isolated via MACS Miltenyi Biotec $CD4^+CD62^+$ T Cell Isolation Kit II mouse following manufacturer's protocol (Auburn, CA). Naive $CD4^+$ cells were plated on α-CD3 (5 μg/mL) coated plates. RPMI-1640 supplemented media with soluble α-CD28 (1 μg/mL) 10% FBS, 1% Penicillin/ Streptomycin, 1% Glutamax, 1% Hepes, 0.5% Non-Essential Amino Acids (NEAA), 0.5% Sodium pyruvate, 50 μM BME, and IL-12 (10 ng/mL) or TGFβ (5 ng/mL) was added in the cultures to polarize towards Th1 or Treg cells, respectively. After 3 days, cells were collected and stained for $CD4^+$, IFNγ, and FoxP3 using Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA).

Dyes, recombinant proteins and antibodies

The antibodies used were as follows: Ly6C-FITC (AL-21), CD68-PE (FA11), IL-17A-PE (TC11-18H10), CD45-PerCP (30-F11), CD11b-PB (M1/70.15), IFNγ-eFluor 450 (XMG1.2), CD4-PerCP (L3T4), F4/80-APC-eFluor780 (BM8), Foxp3-PE (MF23), CD3-APCCY7/eFluor780 (17A2), Ly-6G-PE (1A8), anti-mCCR2- FITC (R&D Systems), and anti-mouse CD16/CD32 (The Lymphocyte Culture Centre, UVA, Charlottesville, VA). To distinguish between live and dead cells, Viability Live Dead-ef650 (eBioscience, San Diego, CA) was used. Recombinant proteins used were as followed: mouse IL-4, IL-13, IL-12, IFNγ, TGFβ were purchased from PeproTech (Rocky Hill, NJ). LPS was purchased through Sigma-Aldrich (St. Louis, MO).

In vitro migration assay

A migration assay was performed using 5-*μ*m 24-well plates (Costar). Recombinant 50 ng/mL of CCL2 in 600uL of 1% FBS in RPMI-1640 medium (PeproTech (Rocky Hill, NJ.) was placed in the low chamber. A total of 0.5×10^6 *Stat4*$^{-/-}$*Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ splenocytes in 100 *μ*l of 1% FBS in RPMI-1640 medium were placed on the upper wells of Transwell membranes. Plates were incubated for at 37°C and 5% CO_2 atmosphere. After 3 hrs of incubation the upper wells were carefully removed and migrated cells in the bottom wells were collected, stained with Trypan-Blue, and counted under light microscope. Next migrated cells were stained with a combination of Abs against CD11b, CD4, and F4/80 and the percentage of migrated $CD4^+$, $CD11b^+$ and $CD11b^+/F4/80^+$ cells was determined using

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flow cytometry. The rate of cell migration was expressed as a migration index (%): (number of emigrated cells toward $CCL2^+$ wells divided by the number of migrated cells in control CCL2-negative wells.

Statistical Analysis

Data were analyzed by Graphpad Prism6, comparisons were made using unpaired Student's or Mann-Whithey 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 has limited effects on T helper development during atherosclerosis

To investigate the role of STAT4 in atherosclerosis, we generated *Stat4*-deficient $Apoe^{-/-}$ ($Stat4^{-/-}Apoe^{-/-}$) mice. STAT4 is one of the key transcription factors that regulates the balance between Treg, Th1, and Th17 cells[7]. Therefore, we first tested the impact of STAT4 deficiency on the ability of *Stat4*-deficient $CD4^+$ cells to differentiate into IFNγ-expressing Th1 cells and Tregs under Th1 and Treg polarizing conditions *in vitro* and in atherosclerosis-prone conditions in $Apoe^{-/-}$ mice. *In vitro*-generated Th1 cells from $Apoe^{-/-}$ mice demonstrated a significant expression of IFNγ, while STAT4-deficiency resulted in a 90% reduction in Th1 cell differentiation (**Fig.1A)** confirming a critical role of the IL-12/STAT4 axis in IFNγ induction *in vitro*[20;23]. In contrast, there were no significant differences between percentage (**Fig.1B-C**) or numbers of $IFN\gamma^+CD4^+$ cells from spleens ($Stat4^{-/-}Apoe^{-/-}$ $1.0\pm0.3\times10^6$ vs $Apoe^{-/-}$ $1.2\pm0.1\times10^6$ cells) and peripheral lymph nodes ($Stat4^{-/-}Apoe^{-/-}$ $1.8\pm0.3\times10^5$ vs $Apoe^{-/-}$ $1.5\pm0.3\times10^5$ cells) of $Stat4^{-/-}Apoe^{-/-}$ and $Apoe^{-/-}$ mice by flow cytometry (**Fig.1B-C**). We also detected no difference in *Ifnγ* expression in the spleens by RT-PCR analysis (**Fig.1D**).

STAT4 might also alter the homeostasis of Tregs, which have been demonstrated to play an important atheroprotective role[24]. To test the potential impact of STAT4-deficiency on Treg differentiation, we examined Treg differentiation under Treg-polarization conditions *in vitro* and compared *Stat4*-deficient Tregdifferentiation with that of the control $Apoe^{-/-}$ mice. STAT4-deficiency allowed more robust Treg differentiation of $Stat4^{-/-}Apoe^{-/-}$ $CD4^+$ cells in comparison with $Apoe^{-/-}$ cells **(Fig.1E**) supporting a role of STAT4 in Treg differentiation *in vitro*. Unexpectedly, no differences in $Foxp3^+$ Treg frequency (**Fig.1F and G**) or numbers were found within the PLNs ($Stat4^{-/-}Apoe^{-/-}$ $1.1\pm0.1\times10^6$ ± vs $Apoe^{-/-}$ $1.4\pm0.1\times10^6$ cells, n=12-15) and spleens ($Stat4^{-/-}Apoe^{-/-}$ $2.4\pm0.4\times10^6$ ± vs $Apoe^{-/-}$ $3.0\pm0.3\times10^6$ cells, n=11-15) of $Stat4^{-/-}Apoe^{-/-}$ and $Apoe^{-/-}$ mice (**Fig.1G**). Thus, the abolishment of STAT4 supports Treg formation and reduces Th1 cell differentiation *in vitro,* but has no effects on Treg and Th1 cell development during atherosclerosis. Interestingly, these results suggest an existence of additional pathway(s) that contributes to the Th1 cell development in the absence of STAT4 signaling upon atherosclerotic conditions *in vivo*.

Evidence suggests a requirement of STAT4 for the development of Th17 cells[6]. It has also been shown that the development of atherosclerosis is accompanied by increased

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differentiation of IL-17-expressing cells including Th17 and $\gamma\delta^{+}$IL-17^{+} T cells[21]. To determine whether STAT4-deficiency results in attenuated levels of Th17 cells, we analyzed the mRNA expression of *Il17a* and conducted FACS analysis of splenic cells and found no difference in the percentage of IL-17A^{+}CD4^{+} cells (Suppl.Fig.1) or mRNA expression of *Il17a* in the spleens between *Stat4*$^{-/-}$*Apoe*$^{-/-}$ and $^{-}$*Apoe*$^{-/-}$ mice (**Fig.1H**).

Stat4$^{-/-}$ deficiency has no effects on M1 and M2 macrophage polarization, but attenuates macrophage activation and pro-inflammatory cytokine production, as well as macrophage migration to CCL2

Since STAT4 expression was previously reported for activated monocytes, we next sought to test whether STAT4-deficiency affects MΦ M1 and/or M2 polarization and the functional activity of M1 and M2 MΦs. Upon M1 polarizing conditions, *Apoe*$^{-/-}$ MΦs properly polarized to the M1 phenotype based on the upregulation of iNOS (**Fig.2A**) and CCL2 (Suppl.Fig.2). Interestingly, *Stat4*$^{-/-}$*Apoe*$^{-/-}$ MΦs also successfully polarized into M1 MΦs and expressed similar levels of iNOS compared to *Apoe*$^{-/-}$ MΦs (**Fig.2A**). M2 polarizing conditions effectively induced the differentiation of both *Stat4*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ MΦs towards the M2 phenotype based on the expression of well-known M2 markers such as CD206, Arg1, and Fizz (gene expression: *Mrc1, Arg-1, Retnla, respectively*). Thus, STAT4-deficiency does not affect the differentiation of MΦs to either M1 or M2 phenotype.

To further examine the potential impact of STAT4-deficiency on bone-marrow-derived MΦ activation, we examined the production of several cytokines and the chemokine CCL2 upon LPS stimulation supported by IL-12 and HK-MRSA, are well-known stimuli for cytokine production by MΦs. We also sought to confirm that the differences between wild type and *Stat4*$^{-/-}$ were the same when even the ApoE mutant allele was not present. As demonstrated in Fig.2B, STAT4-deficient MΦ displayed attenuated production of several cytokines including TNFα, IFNγ, IL-10 and chemokines CCL2 and CXCL10. Next to further get insight to an impact of STAT4-deficiency on MΦ functions, we examined the activation status of splenic CD11b^{+} MΦs in the response of LPS, a common MΦ activator. After LPS stimulation, *Stat4*$^{-/-}$*Apoe*$^{-/-}$ MΦs demonstrated reduced expression of the co-stimulatory molecule CD86, and the early activation marker CD69 compared to *Apoe*$^{-/-}$ MΦs, suggesting that STAT4-deficiency affects TLR4-dependent MΦs activation (**Fig.2C**).

While the results obtained from bone-marrow differentiated STAT4-deficient MΦs clearly indicated defective activation and pro-inflammatory cytokine/chemokine production upon stimulation, we also sought to test whether peritoneal MΦs that is another source of MΦs will display similar behavior. We analyzed the production of several cytokines and chemokines in peritoneal MΦs isolated from *Stat4*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice. STAT4-deficient peritoneal MΦs displayed significant reductions of CCL2 (203±20 pg/ml vs 1011±172 pg/ml, *Stat4*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ MΦs, respectively, p=0.05) and therefore decreased ratio of CCL2 expression from *Stat4*$^{-/-}$*Apoe*$^{-/-}$ vs *Apoe*$^{-/-}$ MΦs at the basal levels (**Fig.3A**). Two specific lineages of macrophages, M1 and M2, can inversely affect atherosclerosis development through the release of unique sets of M1 and M2-specific cytokines. M1 MΦs release high levels of IL-12, IFNγ, TNFα, CCL2, and CXCL10 and thus likely play a pro-atherogenic role[25]. In contrast, M2 MΦs serve as anti-inflammatory and

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anti-atherogenic via the release of high levels of IL-10 and TGFβ. While STAT4-deficiency did not influence the capacity of STAT-4-deficient MΦs to differentiate into M1 or M2 MΦs upon M1/M2 polarizing conditions (Fig.2A), *Stat4*$^{-/-}$*Apoe*$^{-/-}$ MΦs displayed decreased levels of IFNγ and CXCL10 (**Fig.3B**) and CCL2 (Suppl.Fig.2) compared to M1 MΦs from *Apoe*$^{-/-}$ mice. It might be possible that reduced IFNγ levels in *Stat4*$^{-/-}$*Apoe*$^{-/-}$ M1 MΦs results in subsequent attenuated levels of CCL2 and CXCL10, the induction of which is strongly regulated by IFNγ. Interestingly, polarized *Stat4*$^{-/-}$*Apoe*$^{-/-}$ M2 MΦs secreted similar levels of IL-10, but reduced levels of CCL2 and CXCL10 compared to *Apoe*$^{-/-}$ M2 MΦs (**Fig.3C**). These data suggest that STAT4-deficiency did not affect on M1/M2 polarization, but had important actions to reduce a proper MΦ activation (CD86 and CD69 expression) and production of several cytokines and chemokines.

Next, to determine potential impact of STAT4-deficiency on migratory responses of splenic CD11b$^+$ myeloid cells to CCL2, we used a transwell assay. As expected, migration of *Apoe*$^{-/-}$ CD11b$^+$F4/80$^+$ macrophages was detected in response to recombinant CCL2 (**Fig. 4A, B**). However, the percentage of migrated STAT4-deficient CD11b$^+$F4/80$^+$ MΦs was decreased (**Fig.4A**). Similarly, the index of migration toward CCL2 was elevated for *Apoe*−/− CD11b$^+$F4/80$^+$ MΦs (**Fig.4B**). In contrast, we found no increase in the index of migration for *Stat4*$^{-/-}$*Apoe*$^{-/-}$ CD11b$^+$F4/80$^+$ MΦs (**Fig.4B**). To get further insight into a potential mechanism of changes in responsiveness to the CCL2-mediated migration; we examined the expression of CCR2, the chemokine receptor that recognizes CCL2. As demonstrated in **Fig. 4C-D**, we detected reduced levels of CD11b$^+$F4/80$^+$ MΦs expressing CCR2 isolated from spleen and blood of *Stat4*$^{-/-}$*Apoe*$^{-/-}$mice. Altogether, these results indicate that STAT4 deficiency attenuates CCR2 expression and the subsequent migration of CD11b$^+$F4/80$^+$ MΦs towards CCL2.

STAT4-deficiency reduces the development of atherosclerosis in Stat4$^{-/-}$Apoe$^{-/-}$ mice

To further test a role of STAT4 in atherosclerosis, we analyzed plaque size in the aortas of 40 week old *Stat4*$^{-/-}$*Apoe*$^{-/-}$ female mice fed a chow diet and detected a major 71% reduction of aortic lesions in comparison with control *Apoe*$^{-/-}$ mice (**Fig.5A**). Lesion area was decreased from 22.8±3.7% to 6.4±0.4% ($p<0.01$). Similarly, we detected a 60% reduction in plaque burden when atherosclerosis was evaluated in male *Apoe*$^{-/-}$ versus *Stat4*$^{-/-}$*Apoe*$^{-/-}$ male (13.2±0.9% and 5.3±0.5%, respectively, $p<0.001$). The percentage of plaque area within the aortic roots of 40-41 weeks old mice fed chow diet was also decreased in *Stat4*$^{-/-}$*Apoe*$^{-/-}$ vs *Apoe*$^{-/-}$ mice (**Fig.5B**). STAT4 deletion also significantly reduced atherosclerosis in western diet fed *Stat4*$^{-/-}$*Apoe*$^{-/-}$ mice vs age and diet-matched *Apoe*$^{-/-}$ mice ($p<0.01$, Suppl. Fig.4, total cholesterol levels 793.5±28.0 mg/dL vs 711.6±15.3 mg/dL, n=10 and 10, respectively). Chow diet fed 40 week old *Stat4*$^{-/-}$ *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice had no difference in body weight, total cholesterol, HDL and LDL levels (Suppl.Table 1). These results clearly demonstrated an important role for STAT4 in atherosclerotic plaque formation in *Apoe*$^{-/-}$ mice.

To further understand the potential mechanism through which STAT4-deficiency reduces plaque formation, we examined the aortic immune composition with a specific focus on T cells and MΦs. STAT4-deficiency was associated with a reduction in local aortic

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inflammation, as revealed by the significant decrease of CD11b$^+$F4/80$^+$ MΦs and highly pro-inflammatory CD11b$^+$F4/80$^+$Ly6C^{high} MΦs within the aortas of *Stat4*$^{-/-}$*Apoe*$^{-/-}$ mice (**Fig.5C**). This proportional reduction in MΦ was also detected in the spleens of *Stat4*$^{-/-}$*Apoe*$^{-/-}$compared with *Apoe*$^{-/-}$ mice (Suppl.Fig.3A). Interestingly, the percentage of Ly6C^{high} pro-inflammatory monocytes, but not the Ly6C$^{low/neg}$ patrolling monocytes was reduced in the blood of *Stat4*$^{-/-}$*Apoe*$^{-/-}$ mice (Suppl.Fig.3B) suggesting that reduced MΦ frequency in the aortas of *Stat4*$^{-/-}$*Apoe*$^{-/-}$ mice is likely due to diminished pool of peripheral blood pro-inflammatory monocytes and attenuated CCR2-dependent migration of monocytes to the atherosclerotic plaques of *Stat4*$^{-/-}$*Apoe*$^{-/-}$ mice.

As STAT4 plays an important role in NK cell biology, we examined the effects of STAT4 deficiency on NK cell content in the aorta and secondary lymphoid tissues and found no difference in the percentage and numbers of NK cells between *Stat4*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice (data not shown). Additionally, the percentage of CD3$^+$ T cells in the aortic total leukocyte population was similar between *Stat4*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice (data not shown). While STAT4 deficiency dramatically reduced the differentiation of IFNγ-expressing Th1 cells and supported Treg differentiation under Th1 and Treg polarizing conditions *in vitro,* to our surprise, we found no difference in the percentage of IFNγ$^+$ Th1 or Treg cells between the aortas of *Stat4*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice by flow cytometry (**Fig.5D**) or RT-PCR analysis (data not shown). Thus, although STAT4-deficiency is clearly protective during atherogenesis, and this phenotype, at least partially, is independent from IFNg$^+$Th1 and Treg cell abundance *in vivo.*

Discussion

The requirement for immune responses at all stages of atherogenesis is well-established; however, the detailed mechanisms that guide leukocyte activation and differentiation are not completely understood[1;4]. An increasing body of evidence suggests that the JAK/STAT signaling is an important regulator of atherosclerosis and tissue remodeling in response to injury[26]. STAT4, a member of the STAT family, plays a critical role in inflammation and several autoimmune diseases including autoimmune myocarditis[27], cystic fibrosis[28], sepsis[29], cardiac allograft vasculopathy[30], allergic airway inflammation[31], experimental autoimmune encephalomyelitis, autoimmune diabetes[17], and high fat diet-induced obesity[32]. To date, the role of STAT4 in atherosclerosis remains unclear. The current study demonstrates for the first time that STAT4 is a pro-atherogenic factor, which regulates atherogenesis, at least partially, in a Th1-IFNγ-independent manner via the modulation of MΦ content within the aortic wall.

Pro-atherogenic Th1 cells require Tbet and STAT4 for their successful differentiation and full spectrum of functions. T cell receptor (TCR)-dependent expression of IFNγ induces STAT1 activation and a subsequent activation of Tbet, resulting in the expression of IL-12Rβ2 chain whereas IL-12 promotes STAT4 activation and is associated to effector functions of Th1 cell differentiation[9;33]. Thus, there are two major pathways that regulate Th1 cell differentiation: TCR/IFNγ/Stat1/Tbet and IL-12/STAT4/Tbet. STAT4-deficient naïve CD4$^+$ cells display attenuated IFNγ$^+$Th1 cell differentiation and increased levels of Foxp3$^+$ Treg generation in response to Th1 and Treg polarizing conditions *in vitro*

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demonstrating the important role of Stat4 in Th cell balance[14;34;35]. We confirm these results and show a 10-fold decrease in the induction of *Stat4*-deficient IFNγ$^+$CD4$^+$ Th1 cells and a 1.7-fold increase in *Stat4*-deficient Foxp3$^+$ CD4$^+$ Tregs *in vitro.* In contrast, similar levels of CD4$^+$IFNγ$^+$ cells are detected within the spleen, PLNs, and the aorta of *Stat4*$^{-/-}$*Apoe*$^{-/-}$ mice, suggesting that mechanisms for the reduction of atherosclerosis in *Stat4*$^{-/-}$*Apoe*$^{-/-}$ mice may be at least partially IFNγ-independent. Several models of experimental colitis and autoimmune myocarditis demonstrate a key role of the IL-12/STAT4 signaling pathway; however the pathology of the disease is IFNγ-independent[35;36,27]. These studies suggest that STAT4 may have an additional impact on the inflammatory processes that are not tightly related to the induction of IFNγ expression in T cells. It is possible that STAT4 affects other properties of Th1 cells, such as TNFα, IL-6 and chemokine production or T cell migration, that play a key role in the progression of many diseases, including atherosclerosis. Interestingly, these data also suggest that Tbet is a major transcription factor that drives IFNγ$^+$ production and Th1 polarization in atherosclerosis.

STAT4 is constitutively expressed in T and NK cells, but upon activation with IFNγ or LPS, monocytes and dendritic cells express high levels of STAT4[15]. Interestingly, STAT4 is phosphorylated in response to IFNα but not IL-12 in human monocytes[15]. Importantly, activated macrophages within synovial tissues obtained from patients with rheumatoid arthritis express high levels of STAT4 suggesting that these myeloid cells might have a role in a prototypical Th1-driven human disease[15]. Importantly, *Stat4*-deficient MΦs have diminished microbicidal activity, low nitric oxide NO, and IFNγ production[37]. In this study, we show that *Stat4*-deficient peritoneal or bone-marrow derived MΦs are capable to differentiate into M1 and M2 MΦs *in vitro,* but *ex vivo* isolated *Stat4*-deficient MΦs release attenuated levels of CCL2, and *in vitro* generated M1 and M2 MΦs produce low levels of IFNγ, CCL2 and CXCL12. These results suggest that STAT4 in MΦs regulates the recruitment of monocytes and T cells via the regulation of specific aortic chemokine microenvironments. To date, mechanisms by which STAT4 modulates MΦ activity are not clearly defined. One of the potential pathways that would be responsible for the low production of pro-inflammatory stimuli by *Stat4*-deficient MΦs could be diminished levels of activation/responsiveness upon stimulation. In line with this hypothesis, we found an attenuated expression of CD69 and CD86 by *Stat4*-deficient MΦs upon in vitro LPS stimulation and low levels of IFNγ and several chemokines. Relevant to our observed phenotype, a study had also revealed an important role of STAT4 in modulation of TLR4-mediated MΦs activation[38].

MΦs are essential components of atherosclerotic plaques and their presence and activation status strongly correlates with the disease progression. Our study demonstrates that the aortas from *Stat4*$^{-/-}$*Apoe*$^{-/-}$ mice contain decreased percentages of CD11b$^+$F4/80$^+$ MΦs within the aortic leukocyte population suggesting that STAT4 is involved in the regulation of MΦ numbers in aortas. Since *Stat4*$^{-/-}$*Apoe*$^{-/-}$ MΦs displayed the attenuated expression of CCR2 and reduced migration towards CCL2, we imply that STAT4 regulates CCL2/CCR2-dependent recruitment of MΦs into the aorta. In line with this data, we have also shown that *Stat4*$^{-/-}$*Apoe*$^{-/-}$ MΦs produce low levels of CCL2, one of the major chemokines regulating monocyte migration to the aorta[39]. This study also highlighted a

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potential role of STAT4 in the regulation of peripheral blood Ly6C^{high} monocytes that are known to be important players in atherosclerosis. Interestingly, we also found low levels of F4/80^{+}CD11b^{+} myeloid cells in the spleen of *Stat4*$^{-/-}$*Apoe*$^{-/-}$ mice. The role of splenic monocytes as a second reservoir for monocyte-derived MΦs in atherosclerotic plaques has been demonstrated[40]. There are several potential mechanisms that may account for a reduced MΦ content in *Stat4*$^{-/-}$*Apoe*$^{-/-}$ aortas including attenuated migration of monocytes from peripheral blood or the spleen or reduced local proliferation within the atherosclerotic vessel. Further studies will be necessary to explore the complex relationship between circulating, splenic monocytes and MΦ content in the aorta.

Conclusion

In this study, we report a pro-atherogenic role for the transcription factor STAT4 in the development of atherosclerosis in *Apoe*$^{-/-}$ mice. STAT4 is an important transcription factor for the Th1 cell generation; however, in this study STAT4-deficiency has limited effects on the Th1 cell content indicating that there are some compensatory mechanisms that support STAT4-deficient Th1 cell development in the conditions of atherosclerosis. Our study demonstrates that STAT4-deficiency primarily affects MΦ activation and CCL2-induced MΦ migration as well as aortic macrophage content. These findings suggest a novel mechanism by which the transcription factor STAT4 influences the development of atherosclerosis in, at least partially, an IFNγ-independent manner through the alteration of immune composition within the aortic wall. This work has important implications for our understanding of mechanisms of atherosclerosis. The results suggest that targeting STAT4 or key downstream targets could provide novel therapeutic opportunities to prevent atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

Apoe	Apolipoprotein E
WD	Western Diet
MΦ	Macrophages
PLN	Peripheral Lymph Node

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- *Stat4*$^{-/-}$*Apoe*$^{-/-}$ deficient mice were generated
- Stat-4 deficiency resulted in the attenuated atherosclerosis
- Reduced atherogenesis was at least partially independent of Th1 cell-derived IFNγ
- STAT4 supports pro-inflammatory macrophage response in atherosclerosis

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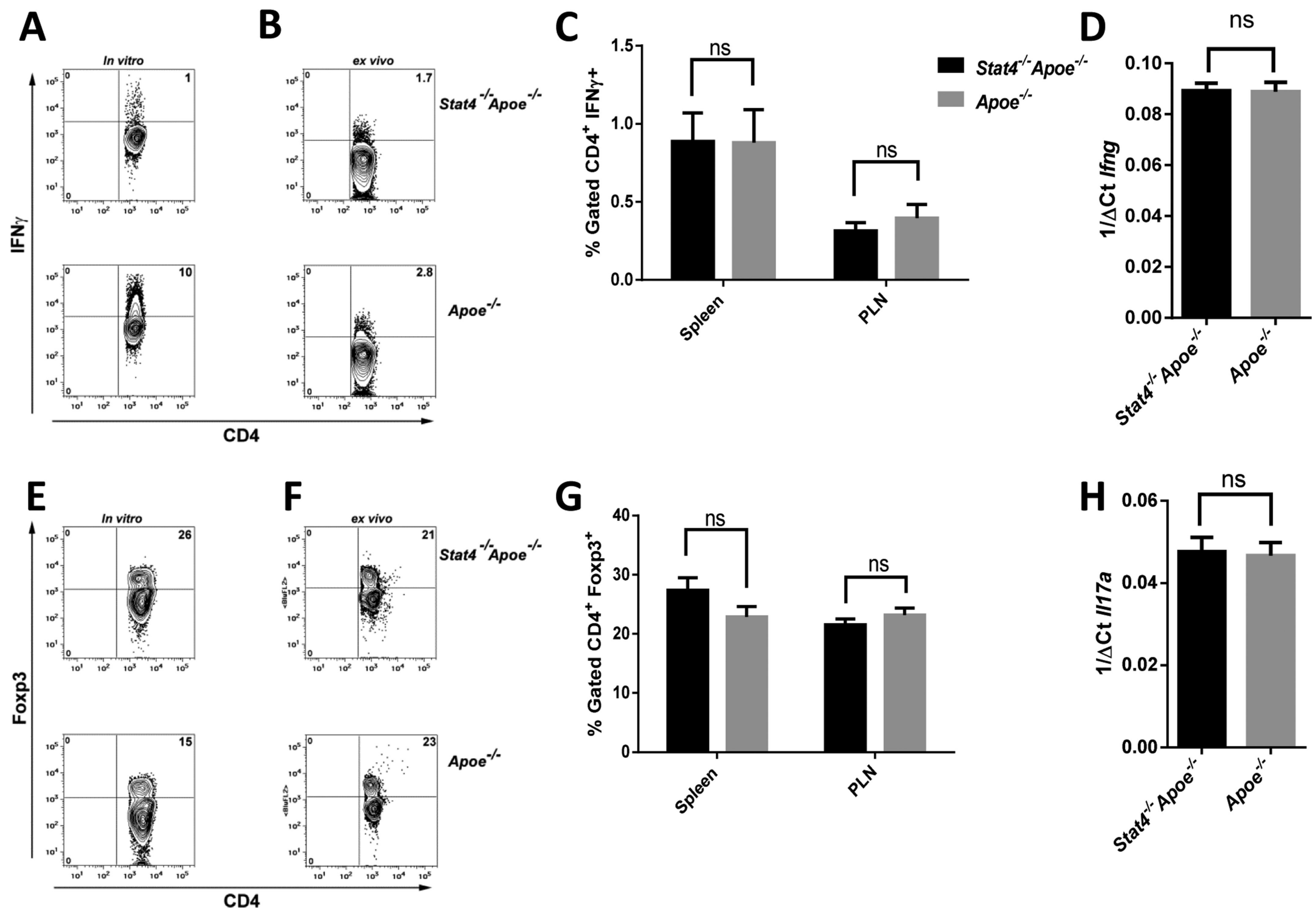

Figure 1. Distinct effects of Stat4 on T cell differentiation and macrophage activation

(A, E) Naïve T cells from *Stat4*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice were cultured under Th1 (A) or Treg (E) polarizing conditions and analyzed for IFNγ and Foxp-3 expression, respectively. Representative FACS plots on gated CD4$^+$ cells from FACS are shown. (B, C, F and G) Spleens and PLNs from *Stat4*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice were analyzed for the expression of IFNγ$^+$ and Foxp3$^+$ by CD4$^+$ cells (n=12). Representative FACS plots on gated CD4$^+$ cells are shown. (C, G) Total percentage of CD4$^+$IFNγ$^+$ and Foxp3$^+$ Tregs in spleens and PLNs (n=12). (D, H) mRNA from spleens was extracted and analyzed for *Ifnγ* and *Il17a* (n=5 and 6). *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.

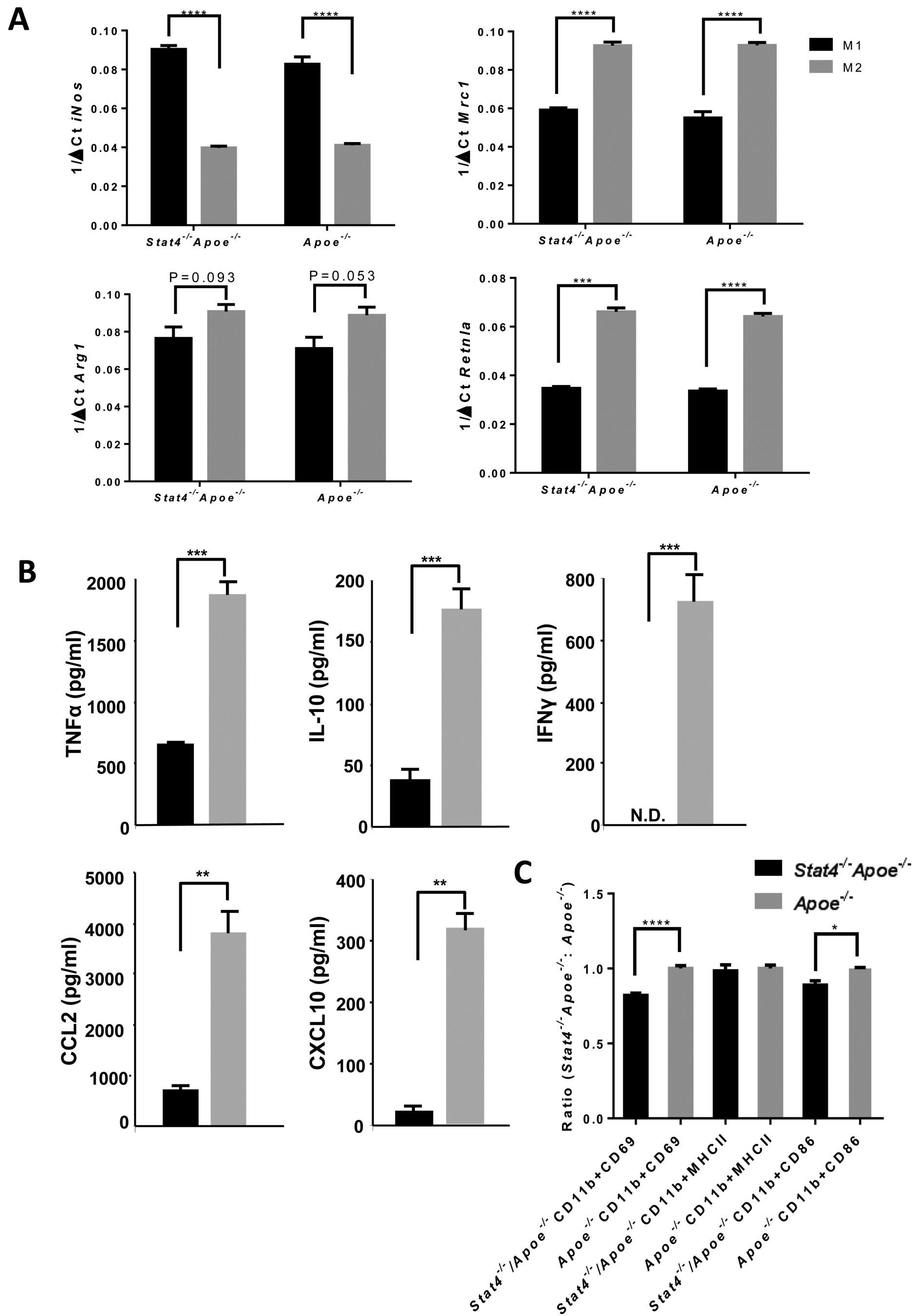

Figure 2. Stat4$^{-/-}$ deficiency has no effects on M1 and M2 macrophage polarization, but attenuates macrophage activation and pro-inflammatory cytokine production, as well as macrophage migration to CCL2

(A) Bone marrow cells were cultured with 10 ng/mL GM-CSF for 7 days, then cultured under either M1 or M2 conditions. PCR was conducted to determine MΦ polarization effectiveness by examination of *iNos*, *Mrc1*, *Arg1*, and *Retnla* expression by M1 and M2 *Stat4*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ MΦs (n=4). (B) *Stat4*$^{-/-}$ (black bar) and wild type (grey bar) BMDM were incubated with heat-killed methicillin resistant Staphylococcus aureus (HK-MRSA) for 24 hours, incubated with IL-12 for 6 hours and stimulated with LPS for 18

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hours. Cytokine concentrations were determined by multiplex assay. (C) Splenic cells were cultured with LPS for 16 hours, and the expression of CD86, I-Ab, and CD69 was determined by flow cytometry. Results show a ratio of percentages of positive CD86, I-Ab, and CD69 MΦs between *Stat4*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ cells (n=6).

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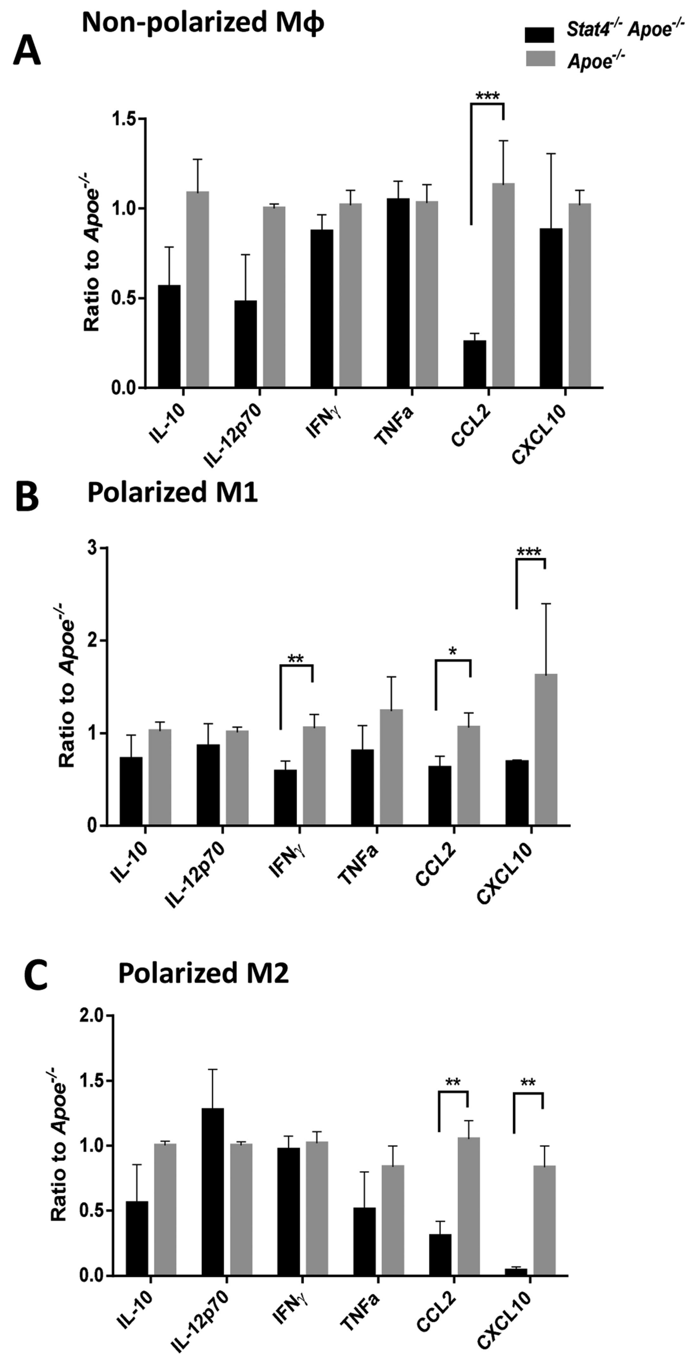

Figure 3. *Stat4*$^{-/-}$*Apoe*$^{-/-}$ peritoneal macrophages display attenuated pro-inflammatory cytokine response

(A-C) Peritoneal macrophages were isolated and cultured under either non-polarizing (A), or (B) M1 or (C) M2 conditions. The media was examined for soluble IL-10, IL-12p70, IFNγ, TNFα, CCL2, and CXCL10. Results show mean±SE as the ratio of *Stat4*$^{-/-}$*Apoe*$^{-/-}$ (black bar) to *Apoe*$^{-/-}$ (grey bar) per independent experiment (n=4). *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.

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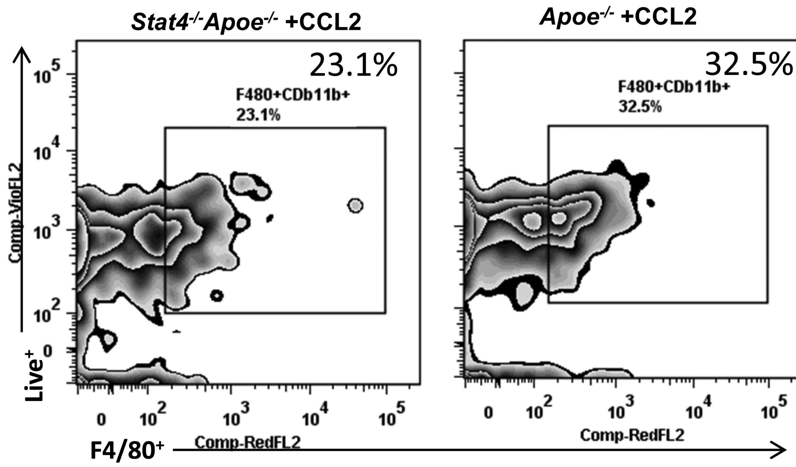
A **CD11b+ gate**



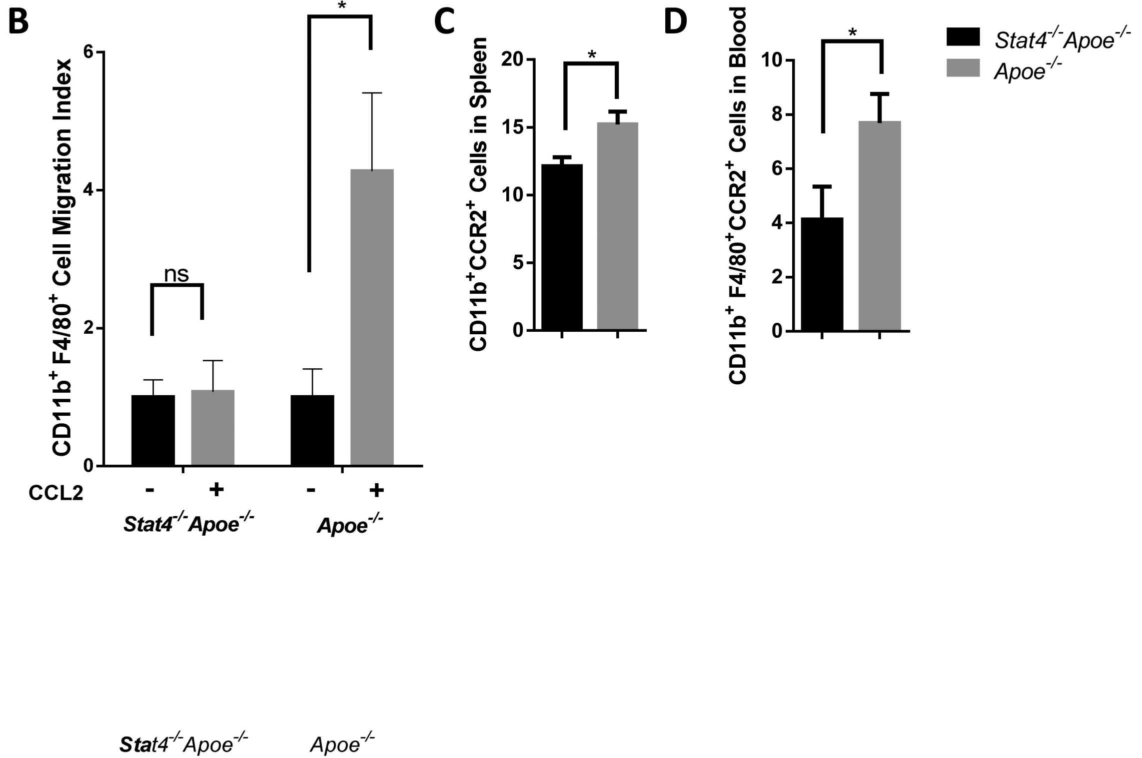


Figure 4. Stat4 deficiency diminishes the migration of CD11b+ F4/80+ splenic macrophages towards CCL2

(A) Representative FACS plots of the transwell migration of CD11b+F4/80+Live+ splenic MΦs from *Apoe*−/− and *Stat4*−/−*Apoe*−/− mice towards 50ng/mL of CCL2. (B) CD11b+F4/80+Live+ migration index with or without CCL2. (C) Ex vivo splenic cells from *Apoe*−/− and *Stat4*−/−*Apoe*−/− mice stained for CD11b+CCR2+Live+ (n=5 and 6). (D) Peripheral blood stained for CD11b+F4/80+CCR2+Live+ cells from *Apoe*−/− and *Stat4*−/−*Apoe*−/− mice (n=5 and 6). * P<0.05, **P<0.01.

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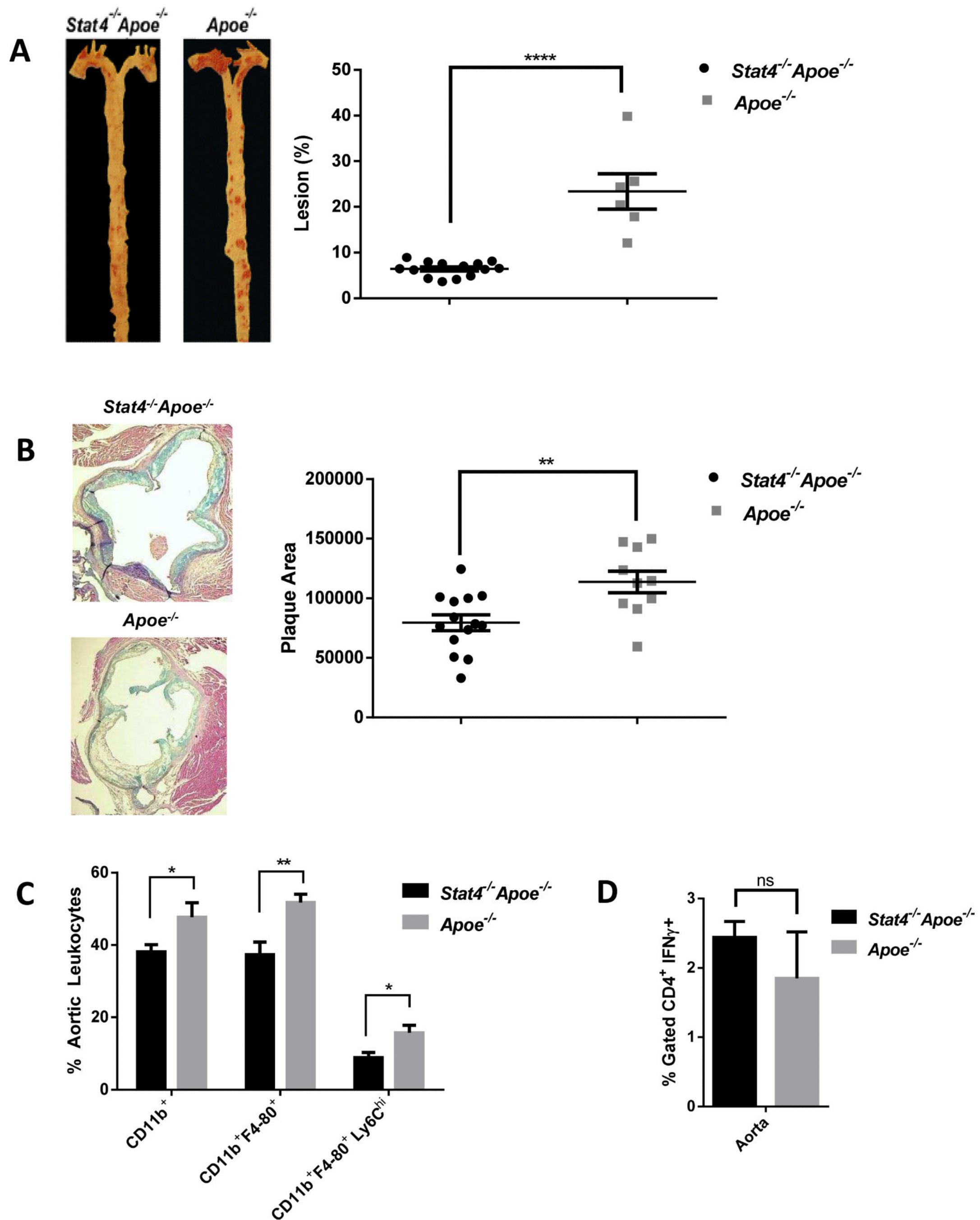

Figure 5. Stat4 deficiency reduces atherosclerosis and alters macrophage but not IFNγ^{+}Th1 cell content in the aortas

(A) Representative en face ORO staining of aortas from aged female *Stat4*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice. Lesion sizes (% of whole aorta) were determined and each symbol represents one animal, horizontal bars represent means. (B) Representative MOVAT aortic root sections from aged female *Stat4*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice fed a CD. Plaque area was determined (mm^2). Each symbol represents one animal, horizontal bars represent means. (C) Aortic cell suspensions were stained with anti-CD11b, -Ly6C, -CD68, -F4/80, and -CD45

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Abs (n=10). (D) The total percentage of $CD4^+IFN\gamma^+$ cells in aortas of *Stat4*$^{-/-}$*Apoe*$^{-/-}$ (black bar) and *Apoe*$^{-/-}$ (grey bar) mice (n=7). * $p<0.05$, ** $p<0.01$

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Table 1

Body weights, total cholesterol, total triglycerides, and HDL and LDL levels in *Stat-4*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice on chow diet.

	Stat4$^{-/-}$Apoe$^{-/-}$	**Apoe$^{-/-}$**
Body Weights, grams	27.69±0.51*	23.60±0.56
Total Cholesterol, mg/dL	272.2±17.7	248.5±19.6
HDL, mg/dL	60.7±6.5	57.2±9.5
LDL, mg/dL	149.4±14.5	130.2±11.7

Data are presented as mean±SEM for BW n=28 and 21 mice and lipids n=10 mice for each genotype. Plasma of fasted 34 week old female mice was analyzed for total cholesterol, triglycerides, and HDL, LDL levels.

*P<0.00001