Nicotinic Acetylcholine receptor alpha 7 stimulation dampens splenic myelopoiesis and inhibits atherogenesis in *Apoe*$^{-/-}$ mice

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Running title: nAChRα7 stimulation inhibits atherogenesis

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Abstract

Background and Aims: Monocyte levels predict cardiovascular outcomes and play a causal role in atherogenesis. Monocytes can be produced in the spleen and track to the atherosclerotic lesion in significant numbers. The cholinergic system has been shown to have anti-inflammatory actions in the spleen. We aimed to explore whether therapeutic stimulation of the nicotinic acetylcholine receptor alpha 7 (nAChRα7) can suppress atherogenesis.
Methods: *Apoe*$^{-/-}$ mice were placed on a Western-type diet and treated with bi-daily injections of the nAChRα7 agonist GTS-21 or vehicle every 2-3 days for 8 weeks.
Results: GTS-21 caused a reduction in atherosclerosis in the aortic arch and proximal aorta. This also resulted in less plaque macrophages. Moreover, GTS-21 reduced the abundance blood monocytes, which was caused by inhibiting inflammatory cytokines and extramedullary hematopoiesis in the spleen, along with splenic monocytes.
Conclusions – Stimulation of the nAChRα7 with GTS-21 reduced atherosclerosis, which was associated with dampened splenic myelopoiesis.

Introduction

Monocytosis is a robust, independent predictor of cardiovascular disease[1] and has been shown to play a causative role in atherosclerosis by homing in to damaged endothelium and subsequently transmigrating into the plaque where they become macrophage foam cells. Genetically depleting monocytes by disrupting M-CSF, an essential monocyte survival factor, results in a gene-dependent decrease in circulating monocytes as well as inhibition of atherogenesis[2]. As well, enhanced monocyte production from classical medullary (i.e. bone marrow) and extramedullary sites (i.e. spleen) have been demonstrated in settings of acute and chronic inflammation[3-6]. With relevance to atherogenesis, Robbins et al, have shown that splenic monocytes track in significant numbers to the plaque[7], highlighting an important role for this organ in the context of atherosclerosis.

The importance of the splenic myeloid reservoir to disease pathogenesis has been shown in myocardial infarction (MI) and sepsis[3, 6]. Preclinical studies suggest that targeting either the production or the release of splenic monocytes in inflammatory settings can significantly improve cardiovascular outcomes[3, 8]. An important mediator of splenic monocyte production post-MI is the sympathetic nervous system (SNS), which can promote extramedullary hematopoiesis and increase the risk of a secondary event[3]. However, targeting the SNS is complicated. A large meta-analysis of 60 trials (>100,000 participants) has suggested that current guidelines should reconsider the 'strength of recommendation for β-blockers post-MI[9].

While there is a deleterious role of the SNS in cardiovascular disease (CVD) [3, 10], the role of the opposing arm – the cholinergic system, is conflicting[11-14]. Interestingly, inhibiting the SNS prevents the activation of the cholinergic system[15]. Thus, we sought to examine the contribution of the cholinergic system in atherogenesis since this potential anti-atherogenic pathway may be perturbed with the administration of β-blockers.

Over the past decade, the physiological mechanism of the 'cholinergic anti-inflammatory pathway' has emerged[16]. Initial evidence describes a role for the nicotinic receptors of the cholinergic system, particularly the nicotinic receptor alpha 7 (nAChRα7). Stimulation of the nAChrα7 in inflamed macrophages reduces the production of TNF-α and has been shown to limit joint inflammation in models of rheumatoid arthritis[16-18]. Moreover, the activation of this pathway has also been shown to be beneficial in acute settings of inflammation such as sepsis, to suppress excessive inflammation mediated via the spleen[19]. Importantly, a selective agonist of the nAChRα7, GTS-21, can effectively reproduce these anti-inflammatory effects *in vivo*[20]. In the context of atherosclerosis, however, this receptor has conflictingly been shown to play a protective[11, 12], deleterious[14] or no[13] role in lesion development, albeit in very differently designed studies with a number of confounding variables making it impossible to draw clear conclusions. Deletion of the nAChRα7 has also been shown to impair macrophage cholesterol handling, contributing to foam cell formation[21].

Thus, given the clear anti-inflammatory potential in acute models and the conflicting reports in the literature, we aimed to test the hypothesis that activation of the nAChRα7, with an agonist proven to incite anti-inflammatory effects *in vivo* (i.e. GTS-21)[20], would reduce the production of splenic monocytes and therefore limit atherosclerotic lesion progression.

Materials and Methods:

Animals:
Animal experiments were approved by the Alfred Medical Research Education Precinct (AMREP) Animal Ethics Committee. *Apoe*$^{-/-}$ mice were purchased from The Jackson Laboratories and bred at the AMREP Animal centre. At 24 weeks of age male *Apoe*$^{-/-}$ mice were placed on a western diet (SF00-219, Specialty Feeds, Aus) for 8 weeks. Mice were injected intraperitoneally with either vehicle (saline) or GTS-21 (4mg/kg) bi-daily every 3 days during western diet feeding. The study was conducted over two separate cohorts separated by ~12 months and data was pooled for analysis. Mice in each cohort were randomised to vehicle or treatment and data analysis was blinded.

Total Plasma Cholesterol:
Total cholesterol levels were measured from the plasma of mice using the Cholesterol E kit (Wako Diagnostics) per the manufacturer's instructions.

White Blood Cell Counts:
White blood cell counts were obtained from blood collected via cardiac puncture immediately following euthanasia. The counts were quantified on a Sysmex XS-1000*i* automated haematology analyser.

Flow Cytometry:
All samples were run and analysed on a BD FACS CantoII flow cytometer. Details of the antibodies used are provided in Supplementary Table 1.
Monocyte populations: Blood collected via cardiac puncture into EDTA-lined tubes and red blood cells (RBCs) were lysed. Cells were washed and stained with CD45, CD115 and Gr1 (Ly6-C/G) at 1:200 dilution for 30 min on ice. Cells were run on the CantoII flow cytometer. Monocytes were identified as CD45^{+}CD115^{+}; Ly6-C^{hi} (Gr1^{+}) and Ly6-C^{lo} (Gr1^{lo}).
Hematopoietic stem and progenitor populations: Splenic cells were obtained by mashing whole spleen through a 40μm strainer and RBCs lyzed. Samples were then stained with a cocktail of antibodies (1:200 dilution, 30mins on ice) before analysis by flow cytometry. Briefly, lineage committed cells were identified as CD45R, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4 and Ly6-C/G, with antibodies against Sca1 and cKit to identify myeloid progenitor cells (Lineage^{-}, Sca1^{-}, cKit^{+}) and HSPCs (Lineage^{-}, Sca1^{+}, cKit^{+}).

RNA Isolation, cDNA synthesis and qRT-PCR:
Total RNA from cells was extracted using QIAGEN RNeasy mini or micro kits and cDNA synthesized using a Tetro cDNA kit (Bioline). qRT-PCR was monitored in real time with a 7500 Fast Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Core Reagents (Agilent Technologies) and normalized to *GAPDH*. All primer sequences can be found in Supplementary Table 2.

Lesion Analysis: H&E, Oil Red O and CD68.
Hearts were dissected after the mice were perfused with saline and frozen in optimal cutting temperature compound (OCT). Serial 6 μm sections of the proximal aorta were prepared. H&E staining was performed as previously described (1). Lipid content was assessed by Oil Red O staining. Macrophage content was quantified by staining for with anti-CD68 (2). All images were captured on an Olympus BX43 microscope and quantification of all images were performed using Adobe Photoshop CC.

Ly6-C and CCR2 immunofluorescent staining

Heart sinus sections were prepared as described above. Sinus sections were fixed with chilled acetone, followed by hydrogen peroxide (in methanol) treatment to quench endogenous peroxidases. Slides were washed in PBS then incubated in Mouse Fc Block (BD Biosciences, USA) solution to prevent non-specific binding. CCR2 (APC, R&D Systems, USA) and Ly6-C (FITC, Biolegend, USA) stains were applied to the sections overnight at 4^{o}C. Slides were washed again in PBS and coverslips were mounted using ProLong™ Gold Antifade Mountant with DAPI (ThermoFisher Scientific, USA). Sections were imaged using a fluorescent Olympus BX-61 microscope. Infiltration of inflammatory monocytes was assessed by immunofluorescent co-localisation of CCR2 and Ly6-C.

Aortic Arch Lipid Analysis:
Lipid content in the aortic arch was measured by *en face* analysis. Dissected aortas were fixed in paraformaldehyde and prior to staining, all fat and connective tissue was removed from the outer layers of the vessel. The aorta was cut longitudinally, stained with Oil Red O followed by washing and mounting on a silicone coated dish. Aortas were viewed on an Olympus SZX10 and captured using Q-Capture Pro 7 (QImaging) software. Quantification of ORO staining was performed off-line using Adobe Photoshop CC.

Statistics:
Data are presented as mean ± SEM (unless stated otherwise) and were analyzed using the two-tailed Student t-test with $P<0.05$ being considered as significant. All tests were performed using the Prism 7 software (GraphPad Software, Inc., La Jolla, CA).

Results

Stimulation of the nAChRα7 reduces atherogenesis

To explore whether pharmacological stimulation of the 'cholinergic anti-inflammatory pathway' via the nAChRα7 inhibits atherogenesis, we fed *Apoe*$^{-/-}$ mice a WTD for 8 weeks and injected them with GTS-21 (twice daily) every 2-3 days. Importantly, no changes were observed in body weight or plasma cholesterol (Figure 1A,B), suggesting that the GTS-21-mediated alterations in lesion formation were not related to altered feeding patterns or whole-body lipid metabolism. Treatment of mice with GTS-21 resulted in a pronounced decrease in the size of the atherosclerotic plaques in the aortic arch and proximal aorta (Figure 1C,D). These experiments confirm that pharmacological targeting of the nAChRα7 receptor can ameliorate atherosclerotic lesions in a well-established pre-clinical model.

GTS-21 promotes a more stable lesion phenotype and reduces blood monocytes

As we saw a robust reduction in atherosclerotic plaque size in the mice treated with GTS-21 (i.e. Fig 1), we explored whether the lesion characteristics were also altered. Consistent with a reduction in lesion size, we also observed a significant reduction in lipid content in the proximal aorta (Fig 2A). Next we assessed the abundance of plaque macrophages which contribute to foam cell formation and proteases that can destabilise lesions. Mice treated with GTS-21 showed a significant reduction in plaque macrophages (Fig 2B). This not only suggests inhibition of atherogenesis but also remodelling of the lesion to a more stable phenotype. As the majority of plaque macrophages arise from infiltrating blood monocytes, and the abundance of circulating monocytes directly influences lesion severity[4, 6], we quantified the abundance of these cells in the circulation. GTS-21 reduce the overall abundance of circulating monocytes, particularly inflammatory Ly6-C^{hi} subset (Fig 2C). No change in blood neutrophils was observed (*data not shown*). Interestingly, we also found less Ly6-C^{+}/CCR2^{+} positive cells in the lesions on GTS-21 treated mice, a reflection of newly

entered blood monocytes[22] (Fig 2D). To determine if GTS-21 directly altered monocyte CCR2 expression, to explain reduced monocyte recruitment to the atherosclerotic lesion we incubated whole blood from WTD-fed *Apoe*$^{-/-}$ mice with escalating concentrations of GTS-21. This revealed a dose dependent decrease in Ly6-C^{hi} CCR2 expression (Fig 2E). These data suggest that GTS-21 dampens myelopoiesis and reduces monocyte CCR2 expression, which could explain the reduced monocyte entry, macrophage content and smaller atherosclerotic plaques.

GTS-21 reduces extramedullary myelopoiesis

The spleen is a major site for monocyte production during atherosclerosis[7] and is a well-known target organ of the cholinergic pathway[23]. Administration of GTS-21 significantly reduced spleen size and splenic leukocyte numbers (Fig 3A,B). GTS-21 administration also reduced splenic monocytes, particularly the atherogenic Ly6-C^{hi} population (Fig 3C). To determine if this was due to decreased production, we investigated the hematopoietic stem and progenitor cells in the spleen. We observed fewer hematopoietic stem cells (HSCs) and myeloid progenitor cell (MPC) populations in the spleens of the treated mice (Figure 2C). nAChRα7 stimulation also reduced inflammatory genes TNF-α, IL-1β, GM-CSFR, and the proliferative marker Ki67, known regulators of myelopoiesis (Fig 2D). Together, these data reveal that treatment with GTS-21 dampens the inflammatory response in the spleen, inhibits extramedullary myelopoiesis, which in turn likely explains the reduction in blood monocytes, contributing to the decrease in atherogenesis.

Discussion

Several clinical and preclinical studies have underscored the importance of circulating monocyte levels and the role they play in contributing to CVD. Controlling the production of these cells can significantly inhibit atherogenesis, lowering the incidence of future CV events. Our findings highlight nAChRα7 as a potential therapeutic target of the cholinergic anti-inflammatory system by reducing circulating monocytes and inhibiting atherogenesis. We show that the spleen is an important site of action for GTS-21 in dampening myelopoiesis, evidenced by reductions observed in splenic HSC, MPC and monocyte populations.

There has been limited studies exploring the role of the nAChrα7 in the context of atherosclerosis, with no clear consensus on the effect on plaque size. Exploring this question using genetic interventions, transplantation of *nAChrα7*$^{-/-}$ bone marrow cells into *Ldlr*$^{-/-}$ mice in one study resulted in either no change in lesion size after 8 weeks of WTD-feeding or a slight reduction after 14 weeks of diet. Using a similar approach another study described no effect on atherogenesis after 7 weeks of diet where only small immature lesions were observed[13]. However, Johansson et al, demonstrated that transplantation of nAChrα7 bone marrow cells into *Ldlr*$^{-/-}$ mice resulted in larger lesions[12]. The reason for the discrepancy between these similar experiments is unclear, but could be due to different methods of transplantation (i.e. a range of 9-14 Grays which could deplete different tissue resident macrophages), different diets (0.25% - 1.25% cholesterol; standard WTD is 0.2% cholesterol) and an unexpected significant decrease in plasma cholesterol[14] in the study describing smaller lesions when the nAChrα7 was deleted. Interestingly, while the study of Johansson et al was unable to provide a robust mechanism, it was suggested that the increased IFN-γ mRNA in the plaque and a trended increase in splenic protein levels could contribute to T cell proliferation, potentiating atherogenesis in the mice receiving *nAChRα7*$^{-/-}$ bone marrow. While this is plausible, the immune cell profiling of the atherosclerotic lesions was unable to corroborate their hypothesis[12].

Based on studies showing robust suppression of inflammation following nAChRα7 stimulation in models of sepsis and endotoxemia that appear to be centred around the spleen[23], we hypothesized that stimulating nAChRα7 may restore the altered myeloid landscape in this important organ, with positive knock-on effects to the lesion. Administration of GTS-21 significantly reduced atherosclerotic lesions in the WTD-fed *Apoe*$^{-/-}$ mice. This is similar to an observation reported by Hashimoto et al, using another nAChrα7 agonist AR-R17779 in *Apoe*$^{-/-}$ mice infused with angiotensin II (Ang II) and briefly placed on a WTD for 4 weeks. In this study, nAChrα7 stimulation also inhibited abdominal aortic aneurysms and increased the survival rate of mice. Stimulating the nAChrα7 was suggested to lower blood pressure and inflammatory genes by downregulating the angiotensin type 1 receptor. However, the lesions of the Ang II infused mice treated with AR-A17779 still presented with an abundance of macrophages, which in this model is challenging to explain.

We found a significant decrease in plaque macrophages in mice treated with GTS-21. This was accompanied with a decrease in extramedullary myelopoiesis. We and others have reported previously that *Apoe*$^{-/-}$ mice produce excess myeloid cells, partly due to extramedullary hematopoiesis[4, 6, 7, 24]. Moreover, we have previously found that increased levels of blood monocytes in these mice results in enhanced recruitment and subsequently larger atherosclerotic lesions, independent of monocyte activation[4]. Consistent with our hypothesis, we observed fewer splenic Ly6-C^{hi} monocytes, the same subset shown by the Swirski group to exit the spleen and enter the atherosclerotic lesion[7]. Stimulation of the nAChrα7 in the spleen could exert anti-inflammatory effects on resident splenic populations, likely macrophages as described by others[16]. We suggest that the anti-inflammatory effects of GTS-21 includes limiting cytokine production which fuels the proliferation of the myeloid progenitor cells, as we also observed a reduction in these cells. Additionally, GTS-21 reduced the expression of CCR2 on blood Ly6-C^{hi} monocytes, which also contribute to fewer plaque macrophages. Thus, the utility of GTS-21 may not just be limited to atherogenesis, as studies inhibiting splenic monocyte reservoirs in other inflammatory settings also result in positive outcomes as alluded to in sepsis, and perhaps more relevant to our mechanism, myocardial infarction.

A limitation to our study, along with the other studies exploring the role nAChrα7 in the context of atherosclerosis, is that the experiments were not designed to determine if there are direct effects of the cholinergic system in the lesion, even though the nAChrα7 appears to be present[12]. Thus, the precise mechanism requires further investigation. However, as excess monocyte production is directly linked to increased plaque macrophages and cardiovascular outcomes, the dampening of this pathway by GTS-21 is likely an important anti-atherogenic mechanism that is promoted by this drug. Overall, our data obtained using a therapeutic approach shows that atherosclerosis in *Apoe*$^{-/-}$ mice can be attenuated by activating the cholinergic system by using the nAChrα7 agonist GTS-21.

Conflict of interest
The authors have no conflicts to report.

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Author contributions

A.A-S, M.K.S.L, J.C.D and A.J.M conceived the idea, designed experiments, interpreted the data and wrote the paper. A.A-S, M.S.K.L, A.W. and M.F performed experiments and analysed the data. All authors read, edited and agreed upon the final version of the manuscript.

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Highlights:

- Stimulation of the nAChRα7 with GTS-21 inhibits atherogenesis
- GTS-21 reduces extramedullary hematopoiesis
- GTS-21 reduces circulating monocytes and lesion macrophages

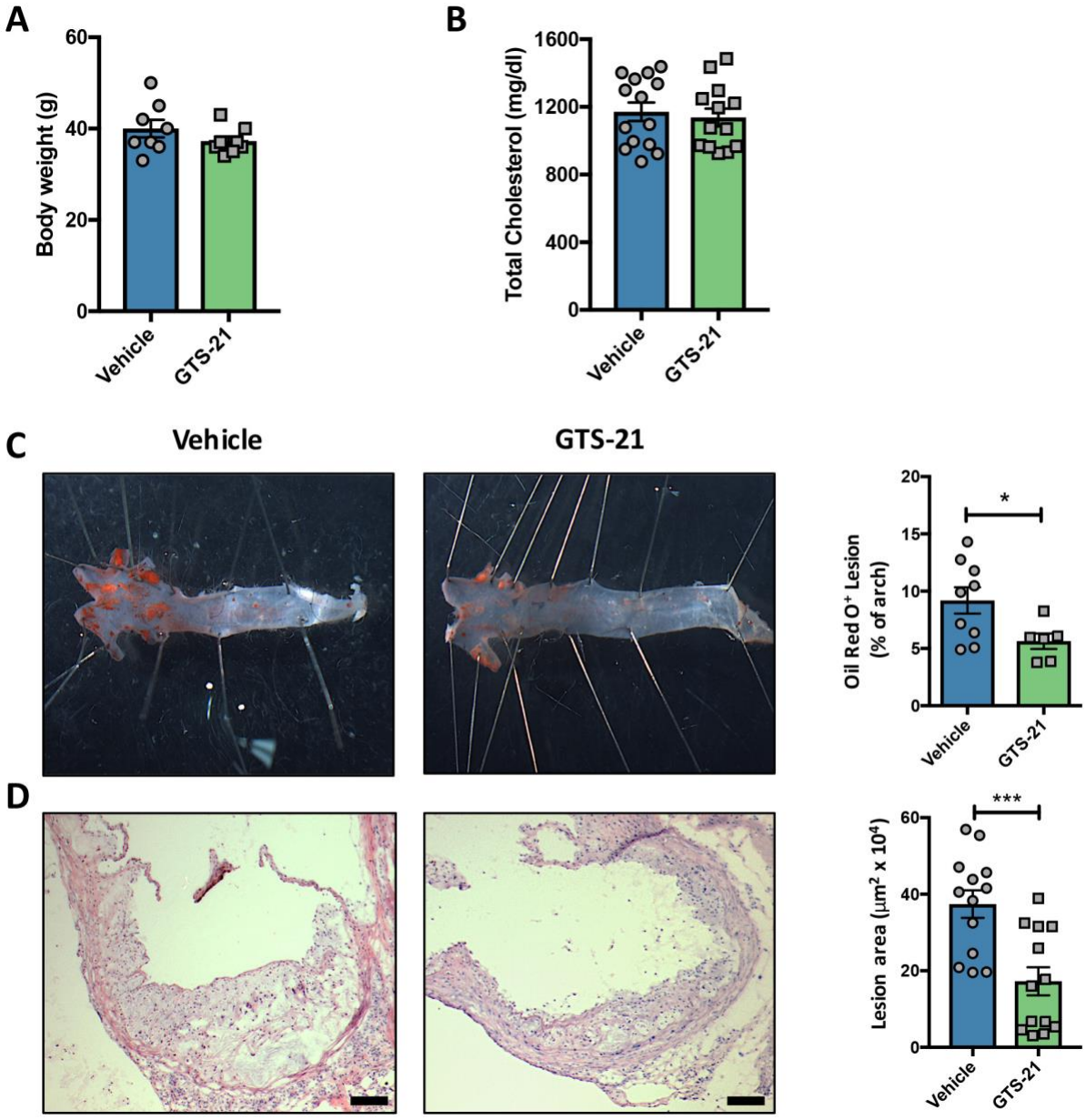

Figure 1. nAChRα7 stimulation reduces atherosclerotic progression. *Apoe*$^{-/-}$ mice were fed a western diet for 8 weeks with or without 4mg/kg GTS-21. **A**) Body weights and (**B**) plasma cholesterol levels were measured. **C**) The aortic arch was stained with Oil Red O to measure lipid abundance **D**) aortic roots were sectioned and stained for plaque area using H&E (Magnification x20, scale bar 100μm). Data are presented as mean±SEM where *p<0.05 and **p<0.01 (Student's t-test). A,B,D) n=13-14/group and C) n=6/group

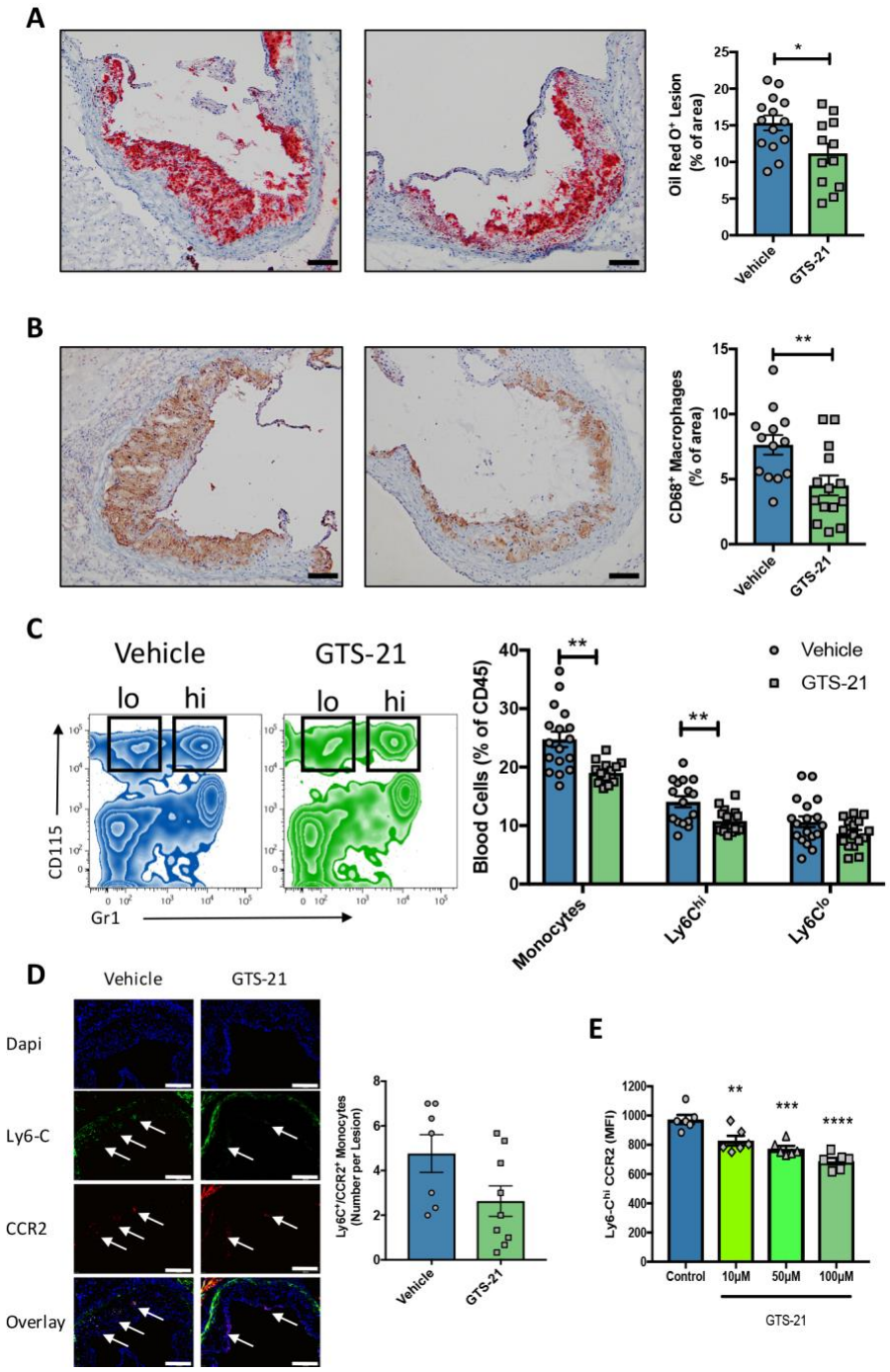

Figure 2. GTS-21 decreases blood monocytes and lesion macrophages. *Apoe*$^{-/-}$ mice fed a western diet for 8 weeks with or without 4mg/kg GTS-21. **A**) Oil Red O staining of the proximal aorta to assess lipid content and **B**) Lesion macrophages were quantified in aortic roots were by immunostaining for CD68 (Magnification x20, scale bar 100μm). **C**) Blood monocytes were detected using flow cytometry (flow cytometry plots and quantified data). **D**) Ly6C+ monocyte infiltration was quantified by staining lesion for Ly6-C and CCR2. **E**) Isolated monocytes from WTD-fed *Apoe*$^{-/-}$ mice were incubated ex vivo with GTS-21 and CCR2 surface expression was quantified by flow cytometry. Data are presented as mean ± SEM where $^{**}p<0.01$ (Student's t-test). A,B) n=13-14/group and C) n=13-18/group. D) n=7-9/group. E) n=6/group.

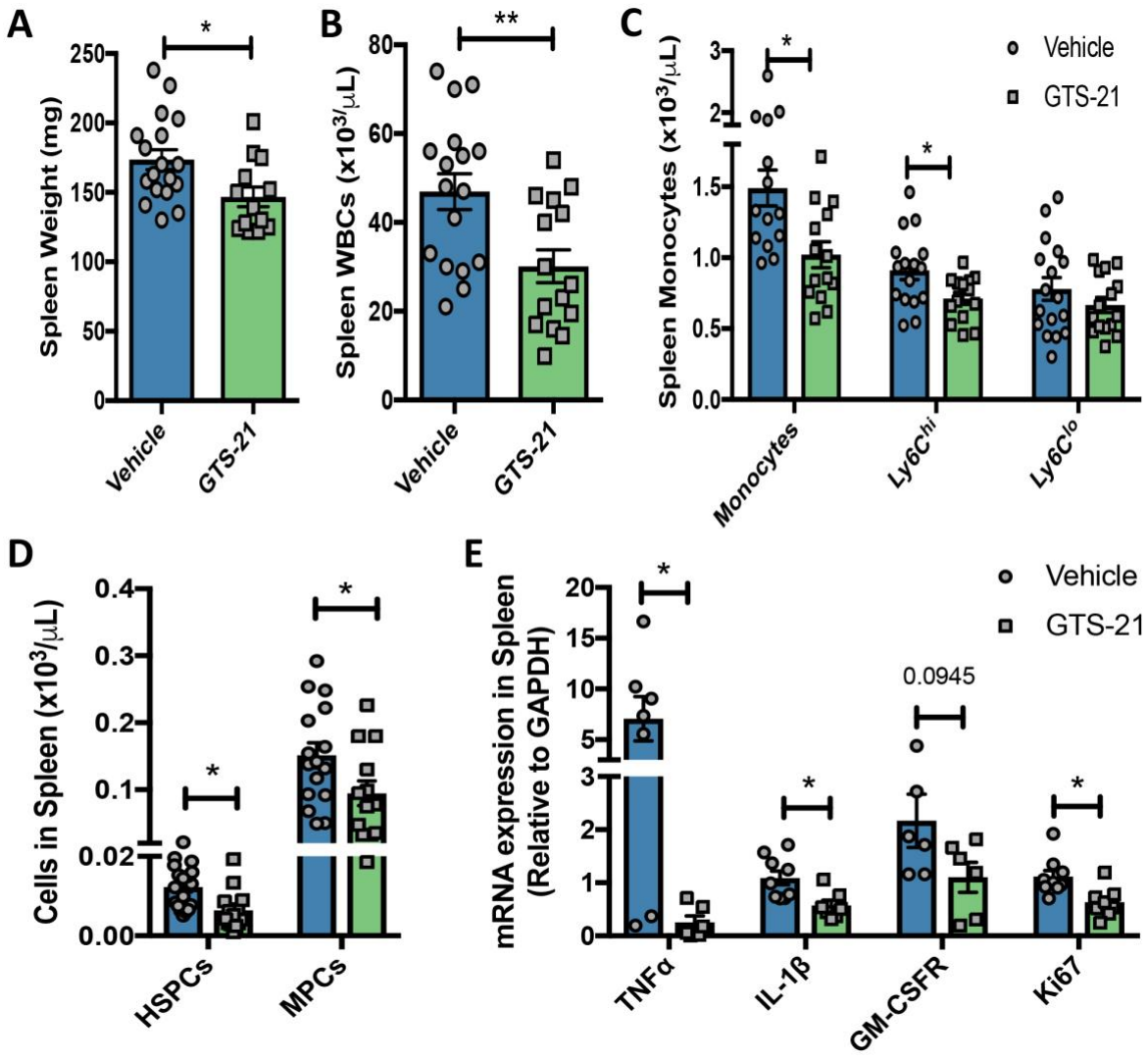

Figure 3. Splenic inflammation and myeloid production is attenuated by GTS-21. *Apoe*$^{-/-}$ mice fed a western diet for 8 weeks with or without 4mg/kg GTS-21. **A**) Spleen weights. **B**) number of splenic WBCs, (**C**) monocytes and (**D**) stem cells were measured using flow cytometry. **E**) mRNA expression of inflammatory and regulatory genes were measured using RT-PCR. Data are presented as mean ± SEM where **p*<0.05 and ***p*<0.01 (Student's t-test). A-D) n=13-18/group, E) n=6-8/ group.

Table S1. Antibody details

Antibodies	Cat #	Clone	Source	Fluorochrome
CD45	103126	30-F11	BioLegend	PB
Gr1	552093	RB6-8C5	BD Pharmingen	PerCP-Cy5.5
CD115	12-1152-82	AFS98	eBioscience	PE
Gr1	553127	RB6-8C5	BD Bioscience	FITC
CD2	11-0021-85	RM2-5	eBioscience	FITC
CD3	11-0033-82	eBio500A2	eBioscience	FITC
CD19	101506	MP19-1	BioLegend	FITC
TER119	11-5921-85	TER-119	eBioscience	FITC
CD45R	11-0452-85	RA3-6B2	eBioscience	FITC
CD8a	553030	53-6.7	BD Bioscience	FITC
CD4	11-0042-85	RM4-5	eBioscience	FITC
Sca1	108120	D7	BioLegend	PB
ckit	105826	2B8	BIOLegend	APC/CY7

Table S2. Primers sequences

Gene	Primer Sequence – forward	Primer sequence – Reverse
GAPDH	5’ – TGA AGC AGG CAT CTG AGG G	5’ – CGA AGG TGG AAG AGT GGG AG
TNFα	5’ – GGT CTG GGC CAT AGA ACT GA	5’ – TCT TCT CAT TCC TGC TTG TGG
IL-1β	5’ – CAA CCA ACA AGT GAT ATT CTC CAT G	5’ – GAT CCA CAC TCT CCA GCT GCA
GM-CSFR	5’ – CAG TGC TTC ATC CTC GTG TC	5’ – AGA GCC AGG AAG CAC ACC
Ki67	5’ – GGG CCT TGG CTG TTT TAC ATT	5’ – GCA GGT TAG CAC TGT TAT GAA AAC