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Myeloid Mineralocorticoid Receptor Transcriptionally Regulates P-selectin Glycoprotein Ligand-1 and Promotes Monocyte Trafficking and Atherosclerosis

Joshua J Man[1,2], **Qing Lu**[1], **M. Elizabeth Moss**[1,2], **Brigett Carvajal**[1], **Wendy Baur**[1], **Amanda E Garza**[3], **Roy Freeman**[4], **Marina Anastasiou**[5,6], **Njabulo Ngwenyama**[5], **Gail K Adler**[3], **Pilar Alcaide**[5], **Iris Z Jaffe**[1,*]

[1]Molecular Cardiology Research Institute, Tufts Medical Center, Boston, MA

[2]Graduate School of Biomedical Sciences, Tufts University School of Medicine, Boston, MA

[3]Division of Endocrinology, Diabetes and Hypertension, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

[4]Department of Neurology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA

[5]Department of Immunology, Tufts University School of Medicine, Boston, MA

[6]Department of Internal Medicine, University of Crete Medical School, Crete, Greece

Abstract

Objective: Mineralocorticoid receptor (MR) activation associates with increased risk of cardiovascular ischemia while MR inhibition reduces cardiovascular-related mortality and plaque inflammation in mouse atherosclerosis. MR in myeloid cells (My-MR) promotes inflammatory cell infiltration into injured tissues and atherosclerotic plaque inflammation by unclear mechanisms. Here we examined the role of My-MR in leukocyte trafficking and the impact of sex.

Approach and Results: We confirm *in vivo* that My-MR deletion (My-MR-KO) in ApoE-KO mice decreased plaque size. Flow cytometry revealed fewer plaque macrophages with My-MR-KO. By intravital microscopy, My-MR-KO significantly attenuated monocyte slow-rolling and adhesion to mesenteric vessels and decreased peritoneal infiltration of myeloid cells in response to inflammatory stimuli in male but not female mice. My-MR-KO mice had significantly less P-selectin glycoprotein ligand-1 (PSGL1) mRNA in peritoneal macrophages and surface PSGL1 protein on circulating monocytes in males. *In vitro*, MR activation with aldosterone significantly increased PSGL1 mRNA only in monocytes from MR-intact males. Similarly, aldosterone induced, and MR antagonist spironolactone inhibited, PSGL1 expression in human U937 monocytes. Mechanistically, aldosterone stimulated MR binding to a predicted MR response

[*]**Corresponding Author:** Iris Z. Jaffe, MD, PhD, Tufts Medical Center, Molecular Cardiology Research Institute, 800 Washington Street, Box 80, Boston, MA 02111, (617) 636-0620, ijaffe@tuftsmedicalcenter.org.

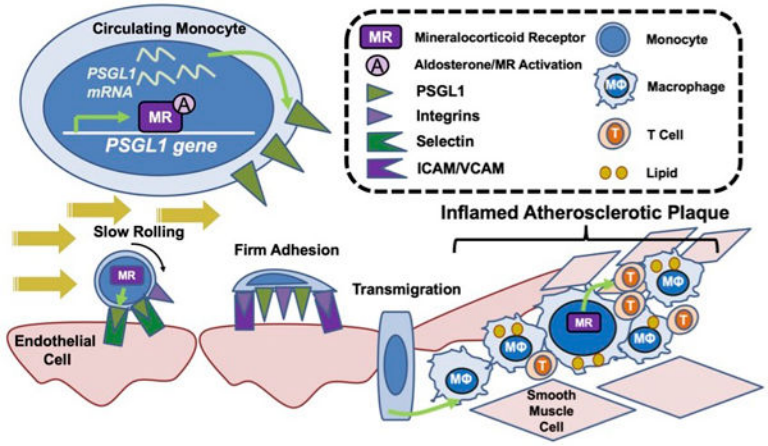
DISCLOSURES

None.

element (MRE) in intron-1 of the PSGL1 gene by ChIP-qPCR. Reporter assays demonstrated that this PSGL1 MRE is necessary and sufficient for aldosterone-activated, MR-dependent transcriptional activity.

Conclusion: These data identify PSGL1 as a My-MR target gene that drives leukocyte trafficking to enhance atherosclerotic plaque inflammation. These novel and sexually dimorphic findings provide insight into increased ischemia risk with MR activation, cardiovascular protection in women, and the role of MR in atherosclerosis and tissue inflammation.

Graphical Abstract



INTRODUCTION

The mineralocorticoid receptor (MR) is a hormone-activated transcription factor, classically activated by the steroid hormone aldosterone, that regulates renal ion channel expression, thereby controlling electrolyte balance and blood pressure.[1] However, elevated aldosterone levels are associated with a 3- to 6-fold increased risk of myocardial infarction or stroke, independent of blood pressure.[2,3] Atherosclerosis is a chronic inflammatory vascular disease, and rupture of inflamed atherosclerotic plaques is the predominant cause of cardiovascular (CV) ischemic events such as myocardial infarction and ischemic stroke. Atherosclerosis is modeled in atheroprone apolipoprotein E-knockout (ApoE-KO) mice in which low-dose aldosterone infusion increases atherosclerotic plaque size and intra-plaque inflammatory cell accumulation.[4] Conversely, MR antagonists decrease CV-related mortality in clinical trials[5,6] and attenuate plaque inflammation in atherosclerotic mouse models.[7–9] Ample data in other models of cardiovascular, renal, or neurologic diseases demonstrate that MR activation correlates with monocyte and macrophage accumulation in injured tissues that contributes to tissue damage and fibrosis.[9–13] These studies provide strong evidence that MR activation promotes tissue inflammation and atherosclerosis without changes in blood pressure, suggesting renal-independent mechanisms that are not fully understood.

Animal studies support a predominant role for MR in the early stages of atherogenesis.[4,14] One of the earliest steps in atherogenesis is leukocyte trafficking, a process initiated when endothelial cells (ECs) become damaged in the setting of traditional CV risk factors, resulting in increased EC expression of adhesion molecules that promote recruitment

of circulating leukocytes into the vessel.[15] Leukocyte trafficking into tissues is a well understood sequential process, beginning with leukocyte rolling via interactions between endothelial selectins and glycoprotein ligands on circulating leukocytes, including P-selectin glycoprotein ligand-1 (PSGL1). Subsequently, firm adhesion is mediated by leukocyte integrins which leads to trans-endothelial migration of leukocytes through the vessel and into tissues. In atherosclerosis, monocytes infiltrate into the sub-intima of the vessel wall where they differentiate into macrophages, scavenge oxidized lipids to form atherosclerotic plaques, and release pro-inflammatory factors that result in chronic vascular inflammation. Pro-inflammatory leukocytes also release matrix metalloproteases that can degrade the fibrous cap of the plaque, thereby causing plaque rupture and vessel thrombosis, the predominant cause of ischemia to downstream organs.[16] Thus, understanding mechanisms driving leukocyte trafficking to induce vascular inflammation is important to mitigating the adverse consequences of atherosclerosis.

MR is expressed in all cells of the vasculature, including ECs,[17] smooth muscle cells (SMC),[18] and leukocytes.[19] Deletion of MR from ECs (but not SMCs) in an atheroprone mouse model attenuated vascular inflammation in a sex-specific manner.[8,20] MR in myeloid cells (My-MR) has been found to promote a pro-inflammatory macrophage phenotype *in vitro*[21] My-MR also contribute to monocyte and macrophage infiltration into injured tissues *in vivo*,[9,11,21–25] but the mechanism remains unknown. One study recently examined the role of My-MR in atherosclerosis, revealing that My-MR-knockout (KO) decreases plaque size and histologic markers of inflammation in a model of late-stage atherosclerosis.[26] However, the role of My-MR in leukocyte trafficking, has never been explored, nor have sex differences been considered. Thus, we explored the impact of My-MR-KO on early atherogenesis, confirming a role in vascular inflammation, and further examined the role of My-MR in leukocyte trafficking, directly comparing these outcomes in male and female littermates to uncover potential sex differences. We demonstrate that My-MR contributes to early plaque inflammation and to leukocyte trafficking with a predominant impact on leukocyte slow-rolling that is more significant in male mice. We identify PSGL1, a critical mediator of leukocyte rolling and atherosclerosis,[27] as a novel MR transcriptional target gene in mouse and human monocytes that is regulated by the MR in a sex-specific manner.

MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding author upon request. For detailed methods, see the online Supplemental Methods.

Study Approval

All mice were handled in accordance with US National Institutes of Health standards, and all procedures were approved by the Tufts University Institutional Animal Care and Use Committee. All human studies were conducted according to Declaration of Helsinki principles. Institutional review boards at Brigham and Women’s Hospital and Beth Israel Deaconess Medical Center (Boston, MA) approved the clinical study procedures, and all subjects provided written informed consent prior to study enrollment.

Mouse Models

MR floxed mice ($MR^{fl/fl}$)[28] were crossed with LysM-Cre mice (The Jackson Laboratory) to generate $MR^{fl/fl}$ Cre^{-} (My-MR-intact) and Cre^{+} (My-MR-KO) littermates. LysM-Cre x $MR^{fl/fl}$ mice were crossed to apolipoprotein E-null (ApoE-KO) mice (The Jackson Laboratory). The resulting LysM-Cre x $MR^{fl/fl}$ x $ApoE^{-/-}$ mice were maintained on standard laboratory diet until 2 months old and then switched to a high-fat diet for eight weeks to model early atherogenesis. For intravital microscopy studies, mice were anesthetized by intraperitoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg) and sacrificed by cervical dislocation at the end of the procedure. For all other mouse studies, mice were sacrificed under anesthesia (4% isoflurane) by terminal blood and tissue harvest.

Aortic Root Histology and Intravital Microscopy

Atherosclerotic plaque histology and intravital fluorescence microscopy (IVM) were performed as described[8] (**Supplemental Videos I-XI)**. To specifically label circulating monocytes, bone marrow monocytes were isolated from male and female My-MR-intact and My-MR-KO mice using a commercial kit (Miltenyi Biotec) and labeled with Rhodamine 6G. Four million monocytes were injected into sex-matched My-MR-intact mice via tail vein and imaged.

Quantifying Plaque and Peritoneal Inflammation by Flow Cytometry

Aortic arch tissue was digested to single cells, and flow cytometry was performed according to the gating scheme in Supplemental Figure IIIA. For all flow cytometry studies, single-stained controls were used to calculate compensation, and fluorescence-minus-one (FMO) controls were used to determine gating.

Acute Peritonitis Model to Quantify Trans-endothelial Migration

Male and female My-MR-intact and My-MR-KO littermates were administered 1 mL of aged 3% Brewer’s thioglycolate solution (Sigma) intraperitoneally. Peritoneal cells were collected 24 hours later by lavage and quantified by flow cytometry.

Quantifying Leukocyte Adhesion Ligand mRNA and Surface Adhesion Ligand Protein Expression

Resident peritoneal macrophages were collected by lavage. RNA was extracted and quantified by RT-PCR with primers are listed in the Supplemental Materials. Peripheral blood leukocytes were treated with anti-CD16/CD32 antibodies (10 μg/mL) and stained with anti-CD11b-APC, anti-CD3ε-PerCP-Cy5.5, anti-CD115-BV421, anti-Ly-6G-APC-Cy7, anti-CCR2-FITC, anti-PSGL1-BV711, anti-ITGA4-BUV563, and anti-LFA1-PE or anti-Ly-6C-PE antibodies (4 μg/mL). Immune cell subsets were defined by the gating scheme in Supplemental Figure IVA. In a separate experiment, MFI of anti-PSGL1-BV711 staining on Ly-$6C^{hi}$ and Ly-$6C^{lo}$ monocytes was quantified.

Quantifying PSGL1 mRNA Expression in Bone Marrow-Derived Monocytes (BM-Mo), U937 Cells, and Human Peripheral Blood Mononuclear Cells (PBMCs)

BM-Mo isolated from male and female My-MR-intact and My-MR-KO mice were treated with 10 nM aldosterone or vehicle for 6 hours. U937 human monocytes were treated with 1 μM spironolactone or vehicle for 30 minutes +/− 10 nM aldosterone or vehicle for 6 hours. RNA was isolated, and mRNA expression was quantified by RT-PCR with primers listed in Supplemental Materials.

ChIP-qPCR

20–25 million U937 cells were treated with 10 nM aldosterone or vehicle (DMSO) for 90 minutes and processed for ChIP using 8 μg anti-human MR antibody (University of Iowa, rMR1–18 1D5) or mouse IgG1 isotype control. MR binding to the first intron of the PSGL1 gene was assessed by quantitative PCR with primers targeting exon 2 of the PSGL1 gene and the promoter of the CTGF gene as negative and positive controls, respectively. Primers are listed in the Supplemental Materials.

Luciferase Reporter Assay

HEK293 cells lacking endogenous MR were co-transfected with: 1) luciferase reporter containing the PSGL1 intronic sequence intact, the PSGL1 intronic sequence with the MR response element (MRE, +728 to +735 from ATG) deleted by site-directed mutagenesis, or the empty control luciferase reporter vector; 2) a human MR expression vector (CMX-hMR) or the control expression plasmid; and 3) a beta-galactosidase reporter to normalize for transfection efficiency. Six hours later, cells were treated with 10 nM aldosterone or vehicle (DMSO). Eighteen hours later, luciferase activity normalized to beta-galactosidase activity was measured, as described.[29] All cloning was confirmed by sequencing.

Statistics

All data were collected and analyzed by sex-, genotype-, or treatment-blinded investigators. Each study was conducted with at least three independent experiments for reproducibility. In each mouse study, at least one mouse from each sex/genotype group analyzed was included in each experiment. Statistical analyses were performed in GraphPad Prism Version 7. Scatter dot-plots and error bars represent the mean ± SEM of biological replicates. Distribution of the data was confirmed by F test for two groups and Bartlett's test for more than two groups. For comparison of two groups, statistical significance was determined by two-sided Student's or paired t test if data had equal variance and by Mann-Whitney or Wilcoxon signed-rank test if data had unequal variance or if the sample size was less than 5. For comparison of more than two groups, statistical significance was determined by two-way or one-way ANOVA with Bonferroni's post-hoc test for multiple comparisons if data had equal variance and by Kruskal-Wallis ANOVA with Dunn's post-hoc test for multiple comparisons if data had unequal variance. Statistical tests are described in each figure legend. Differences were considered statistically significant at $p \leq 0.05$. For data analyzed by ANOVA, significance by sex (red) or genotype (black) or significant interaction between sex and genotype are depicted below the x-axis in bold, and significance by genotype

within each sex by post-hoc multiple comparison testing is depicted with horizontal bars and p-values in the graph.

RESULTS

Deletion of myeloid MR in ApoE-KO mice decreases plaque size in early atherosclerosis without impacting traditional CV risk factors or the number of circulating or splenic leukocytes

To investigate the role of myeloid MR in vascular inflammation in early atherosclerosis, a myeloid-specific MR knockout (My-MR-KO) mouse model was generated using the LysM-Cre driver and crossed with ApoE-KO mice. The cell specificity of Cre-mediated recombination was confirmed by genomic DNA PCR. There was no MR recombination in tissues from Cre- mice. MR recombination was only detected in peritoneal macrophages (Per-Mp) isolated from Cre^+ mice, but not in other tissues that contain few resident myeloid cells including the lung (with relatively high proportion of ECs) and the heart and kidney (in which MR has an established role in CV disease, Supplemental Figure IA). PCR of genomic DNA from sorted peritoneal leukocytes further confirmed MR recombination only in Cre^+ macrophages but not in T or B cells (Supplemental Figure IB). MR mRNA expression was also significantly decreased in both Per-Mp and bone marrow-derived macrophages (BM-Mp) from Cre^+ mice compared to Cre^- littermates (Supplemental Figure IC). Incidentally, MR mRNA expression in Per-Mp was significantly higher than in BM-Mp. Since there is no antibody to specifically quantify MR protein in mice, we show the loss of MR protein function by examining LPS-induced IL-1β mRNA expression, a known target of My-MR.[21] As expected, LPS induced a significant rise in IL-1β mRNA in Per-Mp from Cre^- (My-MR-intact) mice which was significantly attenuated in Per-Mp from Cre^+ (My-MR-KO) mice (Supplemental Figure ID), supporting a decrease in functional My-MR in the mouse model.

ApoE-KO/My-MR-KO mice and ApoE-KO/My-MR-intact littermates were next fed high-fat diet (HFD) for eight weeks, and atherosclerotic plaque size and composition were assessed histologically in the aortic roots from male and female mice (Figure 1A, Supplemental Figure IIA). At eight weeks of HFD, consistent with early atherosclerosis, there was a modest but significant genotype effect with decreased aortic root plaque size in My-MR-KO (Figure 1B) with no affect on plaque lipid, necrotic core, or fibrosis content (Figure 1C-D, Supplemental Figure IIB). This modest change in plaque size was not statistically significant within each sex by post-hoc testing. As expected,[30] female ApoE-KO mice had larger plaques compared to male littermates (Figure 1B). Plaques in male ApoE-KO mice had greater lipid content compared to plaques in females (Figure 1C), but these sex differences were not modified by genotype.

After eight weeks of HFD, traditional CV risk factors and circulating and splenic leukocyte counts were measured. My-MR-KO had no impact on fasting serum glucose or cholesterol, plasma aldosterone, body weight, or blood pressure in either males or females (Supplemental Table I). There was no difference by genotype in the number of total $CD45^+$ leukocytes or $CD11b^+$ myeloid cells nor in the percentage of leukocytes that are T cells ($CD11b^-/CD3^+/CD19^-$), B cells ($CD11b^-/CD3^-/CD19^+$), granulocytes ($CD11b^+/Ly\text{-}6G^+/CD19^-$), or monocytes ($CD11b^+/Ly\text{-}6G^-/CD19^-/F4/80^-$) in the circulation (Supplemental

Table II) or in the spleen (Supplemental Table III). Female spleens had a greater proportion of T cells and smaller proportion of granulocytes and Ly-6C^{hi} monocytes compared to males.

Overall, these data confirm the development of a myeloid-specific MR-deficient mouse on the ApoE-KO background in which MR function is specifically and significantly attenuated in myeloid cells. In this model, My-MR-KO has a modest impact on plaque size in early atherosclerosis despite no impact on traditional CV risk factors nor the number of circulating or splenic leukocytes after HFD exposure.

My-MR-KO decreases the number of macrophages and T cells in atherosclerotic plaques

The impact of My-MR-KO on plaque inflammation was next assessed by quantification of immune cell subtypes within the aortic arch plaques using flow cytometry according to the gating scheme in Supplemental Figure IIIA. There was no impact of genotype on the total number of leukocytes (Figure 1E) or myeloid cells (Figure 1F). However, among myeloid cells, there were significantly effect of genotype with fewer macrophages in the plaques of ApoE-KO/My-MR-KO versus ApoE-KO/My-MR-intact littermates (Figure 1G) with no impact of genotype on the number of granulocytes (Figure 1K) or total monocytes (Figure 1H). While My-MR-KO did not impact the number of Ly-6C^{hi} monocytes (Figure 1I), there were significantly fewer Ly-6C^{lo} monocytes in the plaque, and this difference was significant specifically in males (Figure 1J). There were also significantly fewer T cells in the plaque with My-MR-KO, and again this was significant specifically in males (Figure 1L). The number of B cells was not different by genotype (Figure 1M) and was generally very low across all groups, as expected.[31]

Comparing the sexes independent of genotype, plaques in female ApoE mice had significantly fewer total leukocytes and myeloid cells compared to plaques in male littermates (Figure 1E-F). Among myeloid cells, the sex difference was apparent in granulocytes and total monocytes (Figure 1H,K) with specifically fewer Ly-6C^{hi} monocytes in females (Figure 1I).

Expression of inflammation-associated genes was also measured by RT-PCR in atherosclerotic aortic arches isolated after eight weeks of HFD. There was no genotype difference in mRNA expression of the pro-inflammatory genes interleukin (IL)-1β and IL-6; anti-inflammatory gene IL-10; macrophage proliferation-associated colony stimulating factor 1 receptor (CSF1R) and macrophage scavenger receptor 1 (MSR1); T cell chemokine C-C ligand 5 (CCL5); T cell proliferation-associated genes IL-2 and CD25; and genes for CD4^{+} T cell subset-specific transcription factors T-bet, Gata3, RORγT, and FoxP3 (Supplemental Figure IIC-N).

My-MR contributes to leukocyte trafficking

Since My-MR contributed to plaque inflammation without significant changes in traditional risk factors, proportion of circulating leukocyte subsets, or aortic tissue cytokines, we next examined the role of My-MR in leukocyte trafficking. Male and female My-MR-intact and My-MR-KO mice were treated with TNFα intraperitoneally to activate leukocyte trafficking, and the number and velocity of fluorescently labeled intravascular cells

trafficking along mesenteric vessels were quantified by IVM (Figure 2A, Supplemental Videos I-IV). The number of fast-rolling cells was not different by genotype (Figure 2B). However, the number of slow-rolling cells and firmly adherent cells were significantly attenuated with My-MR-KO (Figure 2C-D), and the change in cell adhesion was specifically significant among males. To specifically quantify changes in monocyte trafficking, monocytes were isolated from the bone marrow of male and female My-MR-intact and My-MR-KO mice and fluorescently labeled *ex vivo*. The cells were then injected into sex-matched MR-intact recipient mice via tail vein after intraperitoneal TNFα administration, as above (Supplemental Videos V-VIII). The injection of *ex vivo* labeled monocytes alone did not induce vascular inflammation in mice treated with intraperitoneal normal saline instead of TNFα (Supplemental Video IX). Again, the number of fast-rolling monocytes was not different by genotype (Figure 2E). However, My-MR-KO significantly decreased the number of slow-rolling and firmly adherent monocytes (Figure 2F-G). Among the subgroups, these genotype effects were significant in male but not female mice with a significant interaction between sex and genotype for monocyte adhesion.

Regarding sex differences independent of genotype, the number of slow-rolling intravascular cells was significantly lower in females (Figure 2C), as shown previously,[8] and slow-rolling and adherent monocytes (Figure 2F-G) were also significantly lower in female versus male mice.

My-MR-KO attenuates trans-endothelial migration of myeloid cells during acute vascular inflammation

Next, the impact of My-MR-KO on trans-endothelial migration of specific leukocyte subsets into the peritoneum in response to inflammatory stimuli was assessed. Leukocyte subsets were quantified by flow cytometry 24 hours after intraperitoneal injection of thioglycollate into male and female My-MR-intact and My-MR-KO mice (Figure 3). The total number of recruited leukocytes and myeloid cells was significantly lower in My-MR-KO versus My-MR-intact mice (Figure 3A-B). Among myeloid cells, there were significantly fewer macrophages (Figure 3C) and granulocytes (Figure 3G) in the peritoneal cavity of My-MR-KO versus My-MR-intact mice, and these genotype effects were significant among male mice. There was no genotype difference in the number of total monocytes (Figure 3D). However, there were fewer Ly-6C^{hi} classical monocytes with My-MR-KO and no difference in the number of Ly-6C^{lo} non-classical monocytes by genotype (Figure 3E-F). Among lymphoid cells, there was no difference in the number of T cells or B cells by genotype (Figure 3H-I), consistent with this being a myeloid-specific deficiency of MR. Overall, female mice had significantly fewer myeloid cells in the peritoneal cavity compared to males (Figure 3B) and this sex difference was significant in the number of macrophages, total monocytes, Ly-6C^{hi} monocytes, and granulocytes (Figure 3C-E,G) with no sex differences in Ly-6C^{lo} monocytes or T cells recruited (Figure 3F,H). Overall, these data reveal for the first time, a role for My-MR in leukocyte trafficking and recruitment of myeloid cells to sites of inflammation, particularly in male mice.

My-MR contributes to basal monocyte/macrophage expression of P-selectin glycoprotein ligand-1 (PSGL1) in mice

Next, to explore potential mechanisms for the role of My-MR in leukocyte trafficking, mRNA and surface protein expression levels of specific chemokine receptors, glycoproteins, and integrins known to be critical for leukocyte trafficking were quantified in cells from ApoE-KO/My-MR-intact and ApoE-KO/My-MR-KO mice (Figure 4A-F). In mRNA isolated from resident Per-Mp, there was no impact of My-MR-KO on macrophage expression of C-C chemokine receptor type 2 (CCR2), E-selectin ligand (ESL-1), or integrin subunits alpha-4 (ITGA4), beta-2 (ITGB2), or alpha-1 (ITGA1). Only P-selectin glycoprotein ligand-1 (PSGL1), an established mediator in leukocyte rolling that contributes to atherosclerotic plaque inflammation,[27] was significantly decreased with My-MR-KO (Figure 4B).

Next, surface protein expression of CCR2, PSGL1, lymphocyte function-associated antigen 1 (LFA1), and ITGA4 were quantified on circulating monocytes ($CD11b^{-}/CD115^{+}/Ly\text{-}6G^{-}$) and T cells ($CD11b^{-}/CD3^{+}$) from male and female My-MR-intact and My-MR-KO mice by flow cytometry using the gating strategy in Supplemental Figure IVA. Surface PSGL1 expression was significantly decreased on circulating monocytes from My-MR-KO versus My-MR-intact littermates (Figure 4G) but not on T cells (Figure 4H). This genotype difference in surface PSGL1 on monocytes was significant only among male mice. When monocytes were separated into two populations based on Ly-6C expression, surface PSGL1 expression on $Ly\text{-}6C^{hi}$ monocytes in male but not female mice was significantly decreased by My-MR-KO (Figure 4I). Surface PSGL1 expression on $Ly\text{-}6C^{lo}$ monocytes, while relatively low compared to $Ly\text{-}6C^{hi}$ monocytes as expected (not shown), was not significantly impacted by genotype in either sex (Figure 4J). However, surface PSGL1 was significantly higher on female $Ly\text{-}6C^{lo}$ monocytes, and there was a significant interaction between sex and genotype in both monocyte populations. Consistent with the mRNA data, there was no genotype difference in surface CCR2 or LFA1 expression on either cell type (Supplemental Figure IVB-E). Surface ITGA4 expression was significantly decreased on T cells (but not monocytes) from My-MR-KO mice, although there was relatively low expression of ITGA4 on T cells compared to monocytes (Supplemental Figure IVF-G). Consistent with the literature,[32] CCR2 expression on monocytes was lower in females compared to males (Supplemental Figure IVB). Overall, mice lacking My-MR had decreased PSGL1 protein expression on circulating monocytes and less PSGL1 mRNA in Per-Mp. In $Ly\text{-}6C^{hi}$ monocytes, the decrease in PSGL1 was significant in males, which is consistent with the decrease in monocyte slow-rolling in males by IVM in Figure 4F.

Aldosterone induces PSGL1 expression in mouse and human myeloid cells in a MR-dependent manner

Since the MR is a transcription factor that can be activated by the hormone aldosterone, we next assessed whether aldosterone regulates PSGL1 mRNA expression in mouse and human monocytes via the MR. First, bone marrow-derived monocytes (BM-Mo) were isolated from male and female My-MR-intact and My-MR-KO mice, cultured in charcoal-stripped media lacking endogenous hormones, and treated with vehicle or aldosterone for 6 hours at a clinically relevant dose (10 nM). Aldosterone treatment significantly increased PSGL1

mRNA compared to vehicle in BM-Mo from male My-MR-intact mice but not from female mice (Figure 5A). BM-Mo from My-MR-KO mice of either sex were not responsive to aldosterone. Next, U937 cells (a male-derived human monocyte cell line) were treated with the MR antagonist spironolactone (1 μM) or vehicle. MR inhibition significantly decreased PSGL1 mRNA expression compared to vehicle (Figure 5B), suggesting that the MR drives basal PSGL1 expression in monocytes. In another experiment, U937 cells were cultured in charcoal-stripped media and then treated with aldosterone with or without spironolactone. As in the mouse monocytes, aldosterone significantly increased PSGL1 mRNA expression compared to vehicle in human cells (Figure 5C). This was prevented by co-treatment with spironolactone, supporting a role for MR in mediating aldosterone regulation of PSGL1 expression in human monocytes.

PSGL1 mRNA was also measured in peripheral blood mononuclear cells (PBMC) collected in a small, randomized crossover clinical study of 8 patients treated with two doses of 100 mg spironolactone eleven hours apart or two doses of placebo at the same time points (Supplemental Figure VA). PBMCs were collected 36 hours after the first dose. Spironolactone treatment significantly reduced PSGL1 mRNA expression in human PBMCs compared to placebo treatment ($p=0.027$) (Supplemental Figure VB). Altogether, the data in Figures 4 and 5 provide *in vitro* and *in vivo* data supporting that MR regulates PSGL expression in mouse and human monocytes.

MR regulates PSGL1 transcription via a MR-responsive DNA binding element in the intron of the PSGL1 gene

To further examine the mechanism by which MR regulates PSGL1, an *in silico* analysis using the PROMO database predicted a putative glucocorticoid receptor (GR) binding site (bases +728 to +735) within the single intron of the gene encoding human PSGL1 (Figure 6A).[33] Enrichment of GR binding to this region was previously demonstrated by ChIP-seq (NCBI Sequence Read Archive ID: SRX2900586). As GR and MR have nearly identical DNA binding domains, they are predicted to bind similar hormone response element sequences in DNA.[34] Thus, we tested whether MR binds to the predicted region within the PSGL1 intron by chromatin immunoprecipitation (ChIP)-qPCR (Figure 6A). Treatment of U937 cells with 10 nM aldosterone for 90 minutes significantly increased MR enrichment versus IgG on DNA containing the first intron spanned by primers (+643 to +765 and +739 to +874) surrounding the putative MR response element (MRE; Figure 6B). As a negative control, MR binding to the second exon of the PSGL1 gene was not enriched with aldosterone treatment (Supplemental Figure VIA). As a positive control, MR binding to the promoter of the gene encoding connective tissue growth factor (CTGF), a known MR target gene in cardiomyocytes and other cells,[35] was enriched with aldosterone compared to vehicle. Regulation of CTGF by MR in U937 cells was further confirmed, with aldosterone significantly increasing CTGF mRNA expression, and this effect was prevented with MR inhibition by spironolactone co-treatment (Supplemental Figure VIB). Thus, activated MR binds to this intronic MR binding-site in the PSGL1 gene in response to aldosterone in human monocytes.

Next, the ability of this MRE to support aldosterone-regulated gene transcription was further tested in HEK293 cells lacking endogenous MR. Cells were transfected with an empty luciferase reporter or a reporter containing the PSGL1 intron sequence including the MRE and co-transfected with an MR expression vector or empty control expression vector. Aldosterone treatment did not impact luciferase activity when MR was expressed with the empty reporter vector lacking the PSGL1 intron (Figure 6C, left). Nor was the reporter vector containing the PSGL1 intron responsive to aldosterone without the MR (Figure 6C, middle). However, aldosterone significantly increased luciferase activity in cells co-transfected with human MR and the reporter vector containing the PSGL1 intron (Figure 6C, right). This MR-dependent aldosterone-induced transcriptional regulation of the PSGL1 intronic sequence was abolished when the MRE (bases +728 to +735) was deleted (Figure 6D). Together, these data demonstrate that PSGL1 is a MR target gene in monocytes that is transcriptionally regulated by aldosterone via MR binding to a MRE in the PSGL1 intron.

DISCUSSION

In summary, this study demonstrates for the first time that the MR in myeloid cells from males directly transcriptionally regulates PSGL1, a mediator of leukocyte rolling, and contributes to leukocyte trafficking and monocyte infiltration into inflamed tissues, including atherosclerotic plaque. The data support a new model in which My-MR activation drives PSGL1 transcription in circulating monocytes, thereby facilitating vascular leukocyte trafficking, trans-endothelial migration, and atherosclerotic plaque inflammation. Specifically, we demonstrate that: 1) In a mouse model of early atherosclerosis, My-MR-KO decreases atherosclerotic plaque size and macrophage and T cell infiltration; 2) My-MR-KO decreases monocyte slow-rolling and adhesion to the mesenteric vasculature by IVM and prevents recruitment of myeloid cells into the peritoneal cavity in response to inflammatory stimuli, predominantly in male mice; 3) My-MR contributes to surface PSGL1 protein expression on circulating Ly-6C^{hi} monocytes from male but not female mice; 4) *In vitro*, aldosterone induces PSGL1 mRNA in monocytes isolated from male but not female mice, in a MR-dependent manner; 5) MR inhibition decreases PSGL1 expression in human monocytes *in vitro* and *in vivo*; 6) In human monocytes *in vitro*, aldosterone enhances MR localization to a predicted MRE within the intron of the PSGL1 gene; and 7) This PSGL1 intronic sequence mediates aldosterone-induced reporter activity in a manner dependent on the presence of the MR and the intact intronic MRE. Altogether, these data suggest that PSGL1 is a novel MR transcriptional target gene in monocytes and that by regulating leukocyte recruitment and plaque inflammation, My-MR may contribute to the clinical association of aldosterone levels with increased CV ischemic events induced by acute rupture of inflamed atherosclerotic plaques. In addition, the attenuated role for My-MR in females may contribute to the relative protection of young females from CV ischemia.

In atherosclerosis mouse models, global pharmacologic MR blockade was previously shown to decrease plaque size and inflammation independent of blood pressure.[7,8] Our lab demonstrated that MR in SMCs does not impact plaque development while MR in ECs contributes to plaque inflammation, specifically in males, by regulating expression of E-selectin and intercellular adhesion molecule-1 (ICAM-1) on endothelial cells.[8,20] Shen et al. recently reported that My-MR-KO decreases aortic root plaque size and Mac-2

histological staining in male LDLR-KO mice with advanced atherosclerosis (16 weeks of HFD).[26] As MR has been implicated predominantly in early atherogenesis,[4,14] here we examined the role of My-MR in leukocyte trafficking and performed our analysis in ApoE-KO mice at an early time point (eight weeks of HFD). Leukocyte accumulation in early atherogenesis is predominantly due to recruitment of leukocytes from the circulation with less impact of intra-lesional cell proliferation or cell death, which plays a larger role in later atherosclerosis.[36] By using flow cytometry to quantify distinct immune subsets in the plaque and directly comparing males and females to reveal sex differences, we add new data demonstrating that My-MR also contributes to plaque accumulation of macrophages among both sexes and of T cells specifically in male mice. Together with published findings, these data now support the concept that MR activation by aldosterone facilitates leukocyte recruitment by regulating expression of PSGL1 in monocytes as well as E-selectin and ICAM-1 in ECs. These findings support an evolving teleological model in which aldosterone acts in an endocrine fashion via MR in multiple cells and tissues to promote survival in response to injury or infection by coordinating renal sodium retention to maintain blood pressure with vascular leukocyte recruitment to fight infection along with smooth muscle-induced wound healing.[37] In fact, patients with primary hyperaldosteronism have a significantly lower incidence of sepsis and septic shock compared to age- and sex-matched control patients with essential hypertension,[38] and aldosterone prevents death from sepsis in a mouse model.[39] This once protective role for the MR in acute injury or infection may have become detrimental in the setting of our modern sedentary lifestyle with high prevalence of risk factors that induce chronic and diffuse vascular injury. Indeed, primary hyperaldosteronism patients also have significantly higher arterial ^{18}F-fluorodeoxyglucose uptake (a marker of vascular inflammation) and very significantly increased risk of CV ischemic events compared to age-, sex-, and blood pressure-matched control patients with essential hypertension.[2,40] CV ischemic events are typically caused by rupture of vulnerable plaques characterized by more inflammation as well as higher lipid and necrotic core content.[16] Although there was no difference in lipid, necrotic core, or fibrosis content with My-MR-KO in our study, these findings are more prominent in later stage atherosclerosis as the study performed at 16 weeks of HFD showed that My-MR-KO reduced necrotic core content in LDLR-KO mice,[26] suggesting a more pronounced effect of My-MR inhibition on late plaque composition in advanced atherosclerosis. Overall, these findings suggest that My-MR deletion confers a more stable plaque phenotype.

PSGL1 is an adhesion ligand that is highly expressed on the surface of circulating Ly-6C^{hi} monocytes, and this cell population is significantly increased in hyperlipidemia.[41] In an *ex vivo* perfusion model using a carotid artery from ApoE-KO mice, An et al. previously demonstrated that Ly-6C^{hi} monocytes roll and adhere to the atherosclerotic endothelium in a PSGL1-dependent manner.[27] Once recruited to the vasculature, monocytes differentiate into macrophages that drive chronic vascular inflammation in atherosclerosis. Indeed, the same authors showed that PSGL1 deletion results in decreased monocyte and macrophage accumulation in plaques, supporting a major role for PSGL1 in monocyte recruitment to atherosclerotic lesions.[27] Consistent with these published data, our findings that My-MR-KO male mice have decreased surface PSGL1 expression on circulating Ly-6C^{hi} monocytes is in line with the finding of decreased monocyte rolling and adhesion by IVM and

decreased macrophage infiltration in the acute peritonitis model and in the chronic plaque inflammation model. While the enzymes that regulate PSGL1 glycosylation, and therefore function, are well-characterized, little is known about regulators of PSGL1 transcription. PSGL1 is negatively regulated by Bcl6 in follicular helper T cell differentiation,[42] and PSGL1 expression in monocytes was found to be upregulated by soluble CD40 ligand and glutamate in HIV infection via c-myc.[43] MR is a hormone-activated transcription factor which regulates gene expression by binding to regulatory regions in DNA, not uncommonly found in the first intron, to promote gene transcription.[44,45] Our data now support that PSGL1 is a direct MR transcriptional target gene in monocytes. This mechanism may be relevant not only to vascular inflammation in atherosclerosis, but also to many other situations in which vascular monocyte recruitment contributes to pathology. For example, it was recently demonstrated in a mouse model of aortic aneurysm induced by high salt diet with deoxycorticosterone (a mouse mineralocorticoid), that PSGL1 deletion attenuated aneurysm formation,[46] supporting the potential that My-MR regulation of PSGL1 may contribute to aneurysm formation in this model.

Regarding other inflammatory mechanism, there was no difference in surface CCR2 expression on circulating monocytes nor in the proportion of circulating or splenic Ly-6C^{hi} monocytes with My-MR-KO, suggesting no impact of My-MR on the ability for Ly-6C^{hi} monocytes to be deployed from splenic reserves and home towards inflammatory site.[47] Although there were fewer macrophages and Ly-6C^{lo} monocytes in the plaques of ApoE-KO/My-MR-KO mice, there was no difference by genotype in the number of total monocytes or specifically Ly-6C^{hi} monocytes in plaques. This was at first unexpected given the relatively higher expression of PSGL1 on Ly-6C^{hi} monocytes compared to Ly-6C^{lo} monocytes[41] and since recruitment of Ly-6C^{hi} but not Ly-6C^{lo} monocytes was decreased in My-MR-KO mice in the acute peritonitis model. One possibility is that by eight weeks of HFD, recruited Ly-6C^{hi} monocytes may have differentiated into macrophages. Indeed, My-MR has been shown to play a role in monocyte polarization and macrophage differentiation,[21] and this would be consistent with the finding of decreased plaque macrophages in ApoE-KO/My-MR-KO mice after eight weeks of HFD. Finally, specifically male ApoE-KO/My-MR-KO mice had fewer plaque T cells after eight weeks of HFD. This suggests a sex-specific contribution of My-MR to plaque T cell accumulation. However, we confirmed that MR was not deleted in T cells and found that aortic arch expression of the T cell chemokine CCL5 was not different by genotype and that T cell recruitment was unaffected by My-MR-KO in the acute peritonitis model. We propose that the decrease in plaque T cells may be secondary to the decrease in myeloid cells. However, the detailed mechanism by which My-MR orchestrates myeloid-T cell interactions in a sexually dimorphic fashion and how this impacts T cell responses in the setting of chronic inflammation requires further exploration.

This study also identified substantial sex differences in leukocyte trafficking, vascular inflammation, and atherogenesis in mice, independent of My-MR. Very few previous studies have directly compared plaque inflammation in the two sexes with almost none comparing them by quantitative flow cytometry. We report here that: 1) Consistent with some prior studies[30], plaques from MR-intact female ApoE-KO mice are larger yet have fewer total leukocytes, myeloid cells, and T cells compared to male plaques; 2) We further demonstrate

that the sex difference in plaque myeloid cells is due to fewer granulocytes, total monocytes, and Ly-6C^{hi} monocytes in plaques from females compared to males; 3) Females also have decreased myeloid cell leukocyte trafficking in response to acute inflammatory stimuli with fewer slow-rolling and adherent monocytes and decreased transmigration of granulocytes, macrophages, and Ly-6C^{hi} monocytes than male littermates; and 4) Females have decreased surface CCR2 protein on monocytes which may contribute to decreased leukocyte trafficking and plaque inflammation.[48] To our knowledge, this is the first flow cytometric study showing sex differences in myeloid cell subsets in atherosclerotic plaques, which are important contributors to atherogenesis and plaque rupture.[49] Similarly, in the acute peritonitis model, females recruited fewer peritoneal monocytes, granulocytes, and macrophages than males with a lack of significant change with My-MR-KO among female mice unlike in their male littermates. This may be due to estrogen-mediated sex differences in EC adhesion molecule expression, which has been previously shown.[8,29] Overall, these data comparing sexes provide novel mechanistic insights that may contribute to the well-described decreased risk of CV ischemic events in pre-menopausal women.

There are a few limitations of this study. First, although LysM-Cre-mediated MR recombination was specific to macrophages, the recombination efficiency was approximately 50%, as in other models using the LysM-Cre mouse.[50] Therefore, the results likely underestimate the impact of My-MR-KO in the mouse model. Also, as mentioned previously, the models of acute vascular inflammation do not perfectly encapsulate the dynamics of leukocyte trafficking in chronic vascular inflammation in atherosclerosis. For example, whereas My-MR-KO was associated with fewer granulocytes in the peritoneal infiltrate with acute peritonitis, there was no difference in the number of granulocytes in the atherosclerotic plaque by genotype after eight weeks of HFD. IVM could not be used to assess whether there is a genotype effect on leukocyte trafficking in atherosclerotic ApoE-KO mice directly due to technical limitations in imaging intravascular cells through the substantial mesenteric perivascular adipose tissue in the ApoE-KO model. There were also several limitations in investigating the sex differences in the effect of My-MR-KO on vascular inflammation and PSGL1 expression. The decrease in plaque macrophage accumulation in females did not correlate with a decrease in monocyte PSGL1 surface expression with My-MR-KO as in their male littermates. This suggests that the benefits of My-MR inhibition in females is independent of PSGL1 and monocyte trafficking and may be mediated by a mechanism distinct from that of males altogether. One possibility is estrogen receptor alpha-mediated inhibition of MR transcriptional activity[8,29] which might occur in young female mice. This has been shown for MR targets in ECs[29] and would be expected to diminish any effect of My-MR-KO specifically in females. As these studies were all performed in young mice, whether the loss of estrogen signaling with aging in female mice may modulate the interaction of sex with My-MR in vascular inflammation in aging mice also requires further investigation. The human study is small and underpowered but shows a significant decline in PSGL1 in patients when treated with spironolactone. Larger studies are needed to confirm this finding. Lastly, we could not perform transcriptional studies in female human monocytes to complement those in males due to the lack of a commercially available female monocyte cell line, and we could not

confirm MR mRNA changes with protein studies in mice due to the lack of a specific anti-mouse MR antibody, known limitations in the field.

Despite these limitations, the novel mechanistic insights from this study have important clinical implications. Increased aldosterone activation in patients with primary hyperaldosteronism or with known coronary artery disease are associated with a substantially increased risk of myocardial infarction or stroke (3–6-fold).[2,3] The prevalence of MR over-activation is likely underestimated since primary aldosteronism is thought to be responsible for up to 25% of cases of treatment-resistant hypertension, and recent data suggests that it is substantially underdiagnosed.[51] Furthermore, obesity incidence is rapidly increasing and increased body mass index is also associated with aldosterone excess.[52] Thus, MR upregulation of monocyte PSGL1 resulting in enhanced vascular leukocyte recruitment may be contributing to CV disease in rapidly growing patient populations. This mechanism may also contribute to the benefits of MR antagonism observed in many animal models and human trials for other conditions including heart failure, ischemic stroke, aneurysm, renal fibrosis, and other pathologies in which inflammation is implicated.[5,6,8,11–14,53] Finally, since MR antagonism decreased PSGL1 expression in human monocytes, this study would predict that MR antagonists would provide a dual anti-inflammatory benefit by inhibiting MR in both ECs and myeloid cells, warranting clinical trials to assess the benefit of MR antagonists in preventing heart attack and stroke.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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J.J.M. designed and conducted experiments, acquired and analyzed data, and wrote the manuscript.

Q.L., M.E.M., B.C., W.B., M.A., and N.N. designed and conducted experiments.

P.A. provided reagents, helped design and analyze studies, and edited the manuscript.

M.A. and N.N. provided assistance in designing and executing experiments.

A.E.G. performed the human PBMC collection.

R.F. and G.K.A. performed the clinical study and edited the manuscript.

I.Z.J. analyzed data and wrote the manuscript.

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NON-STANDARD ABBREVIATIONS AND ACRONYMS

ApoE-KO	Apolipoprotein E-null

BM	Bone marrow
CV	Cardiovascular
EC	Endothelial cell
IVM	Intravital microscopy
KO	Knockout
MR	Mineralocorticoid receptor
MRE	Mineralocorticoid receptor response element
My-MR	Myeloid cell-specific mineralocorticoid receptor
Per-Mp	Peritoneal macrophage
PSGL1	P-selectin glycoprotein ligand-1
TNFα	Tumor necrosis factor alpha

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HIGHLIGHTS

- P-selectin glycoprotein ligand-1 (PSGL1) is a novel mineralocorticoid receptor (MR) transcriptional target gene in monocytes
- Myeloid MR contributes to leukocyte trafficking and trans-endothelial migration in male but not female mice in response to inflammatory stimuli
- Myeloid MR deletion attenuates atherosclerotic plaque inflammation with fewer macrophages in male and female apolipoprotein E-null mice and fewer T cells specifically in males

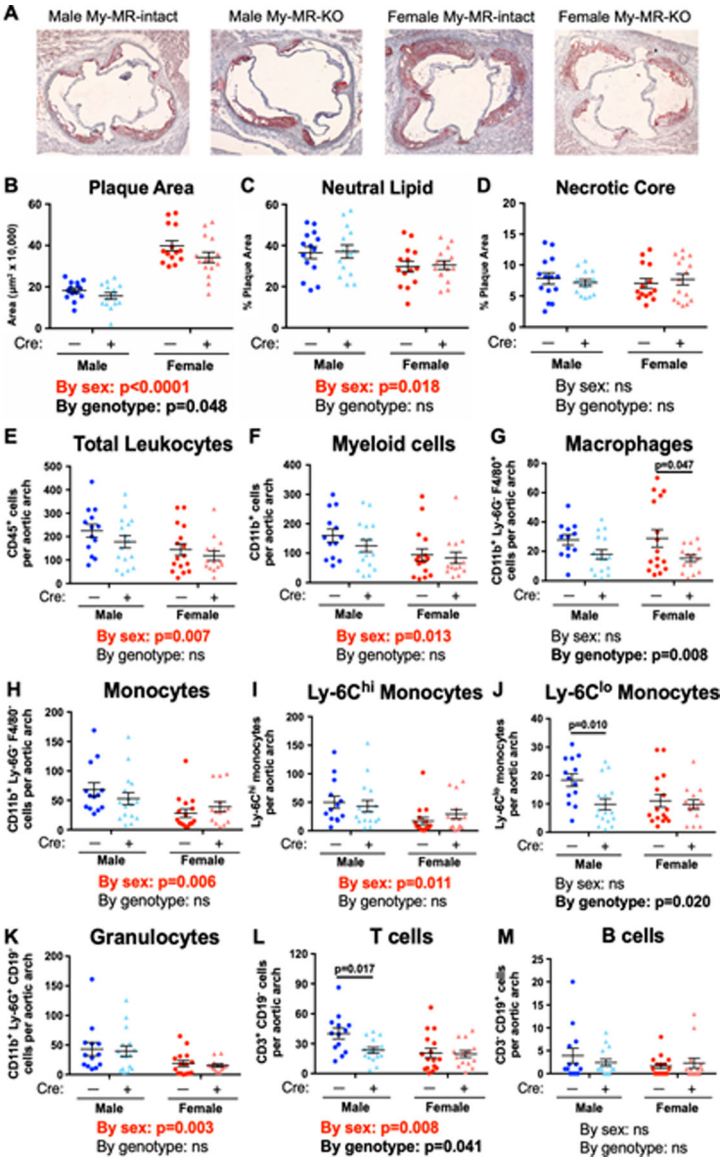

Figure 1. Plaque size and intraplaque macrophage and T cell accumulation is attenuated with My-MR-KO.

Atherosclerotic plaque morphology and inflammation were analyzed in male and female ApoE-KO/My-MR-intact (circles, Cre^{-}) and ApoE-KO/My-MR-KO (triangles, Cre^{+}) littermates treated with HFD for eight weeks by histology and flow cytometry, respectively. (A) Representative Oil Red O/H&E-stained aortic root cryosections. Plaque cross-sectional area (B) and the percentage of plaque area composed of neutral lipids (C) and necrotic core (D) were quantified (n=14–15). (E-M) Single-cell suspensions of atherosclerotic aortic arch tissue were stained and analyzed by flow cytometry. Quantification of total $CD45^{+}$ leukocytes (E) and $CD45^{+}$ $CD11b^{+}$ myeloid cells (F). Of myeloid cells, Ly-$6G^{-}$/$CD19^{-}$/$F4/80^{+}$ macrophages (G), Ly-$6G^{-}$/$CD19^{-}$/$F4/80^{-}$ monocytes (H), and Ly-$6G^{+}$/$CD19^{-}$ granulocytes (K) were quantified. Total monocytes were separated into Ly-$6C^{hi}$ (I) and Ly-$6C^{lo}$ (J) populations. Of lymphoid cells, $CD3^{+}$/$CD19^{-}$ T cells (L) and $CD3^{-}$/$CD19^{+}$

B cells (M) were quantified (n=13–16). Statistical significance was determined by two-way ANOVA with Bonferroni's post-test. ns = not significant.

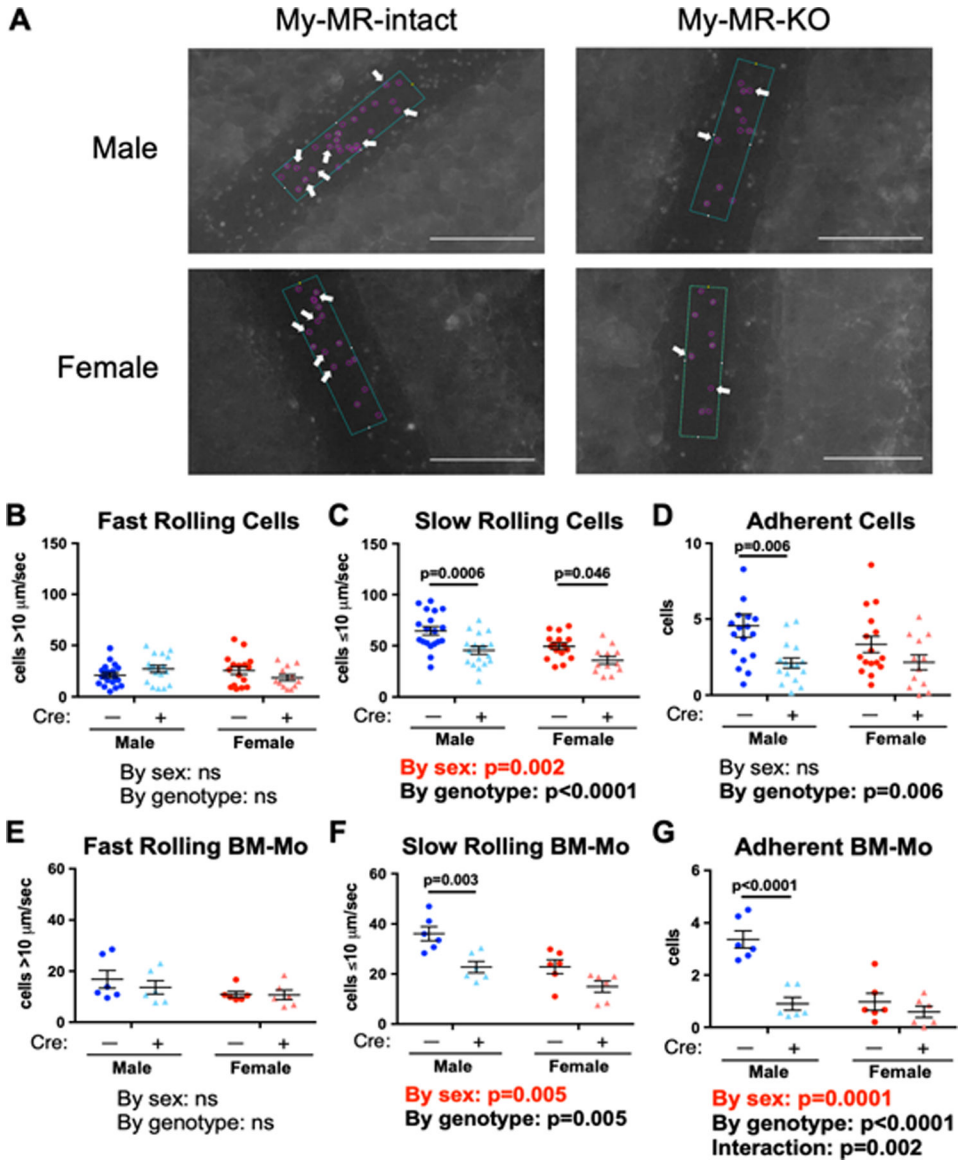

Figure 2. My-MR-KO attenuates monocyte trafficking in vivo.

The number and velocity of fluorescently labeled intravascular cells trafficking in mesenteric veins from male and female My-MR-intact (circles, Cre^{-}) and My-MR-KO (triangles, Cre^{+}) mice four hours after intraperitoneal administration of TNFα was quantified by intravital microscopy. (A) Representative still images depicting Rhodamine 6G-stained intravascular cells (purple circles) at a single frame of a 30-second video recording. Adherent cells are highlighted by white arrows. Scale bar = 50 microns. Fast-rolling (B) and slow-rolling (C) cells were quantified based on a velocity threshold of 10 microns per second. (D) The number of firmly adherent cells were manually counted (n=12–18). (E-G) Bone marrow monocytes (BM-Mo) from male and female My-MR-intact and My-MR-KO mice were isolated, stained *ex vivo* with Rhodamine 6G, and injected into sex-matched wild-type mice via tail vein four hours after intraperitoneal administration of TNFα. Fast-rolling (E), slow-rolling (F), and adherent (G) monocytes were quantified as in B-D (n=6). Statistical

significance was determined by two-way ANOVA with Bonferroni's post-test. ns = not significant.

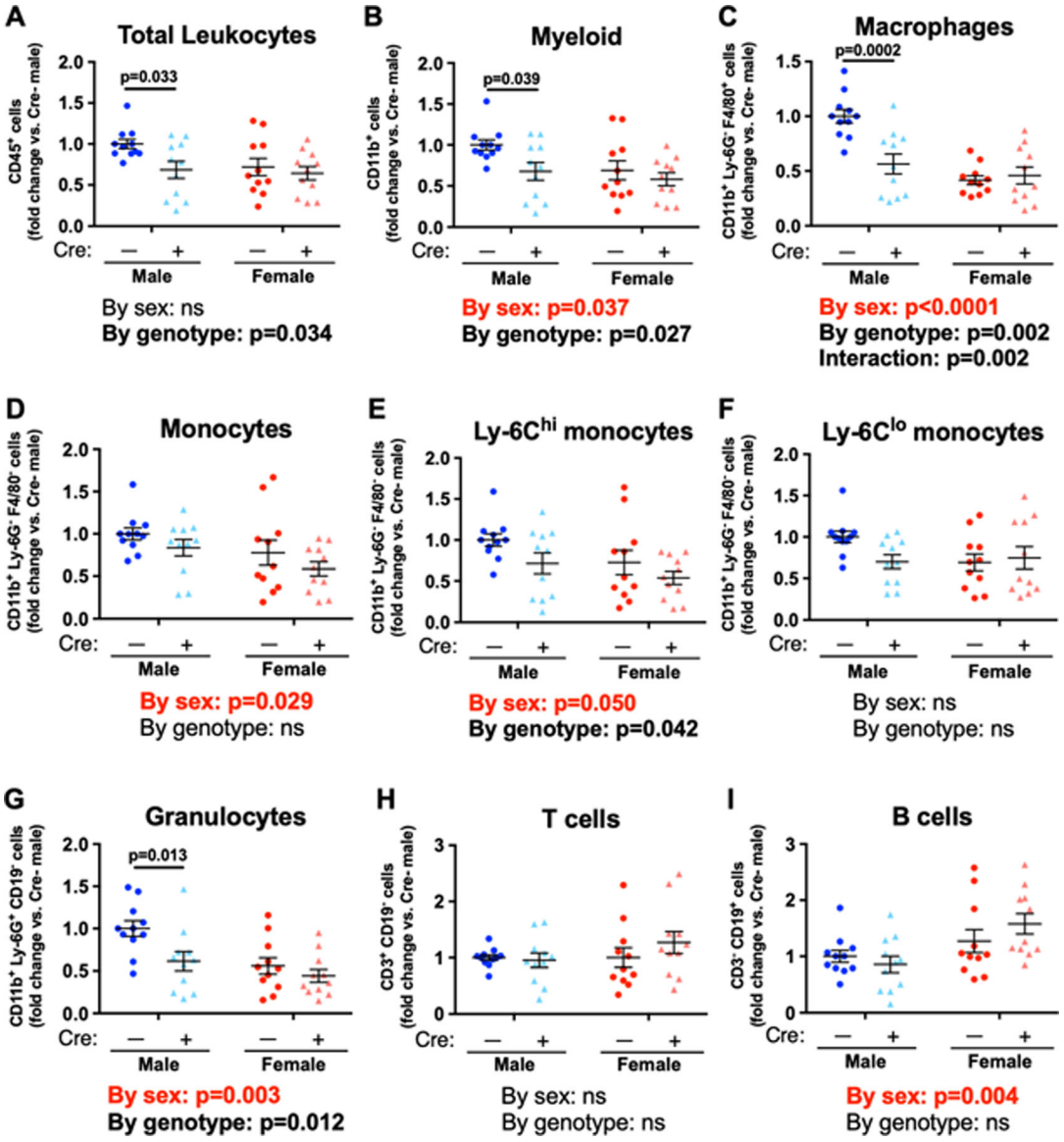

Figure 3. My-MR contributes to myeloid cell recruitment in response to inflammatory stimulus in males.

Leukocytes subsets in the peritoneal cavity of male and female My-MR-intact (circles, Cre^{-}) and My-MR-KO (triangles, Cre^{+}) mice after intraperitoneal thioglycollate administration were quantified by flow cytometry. Total $CD45^{+}$ leukocytes (A) were divided into $CD11b^{+}$ myeloid (B) and $CD11b^{-}$ lymphoid cells. Of myeloid cells, $Ly\text{-}6G^{-}/F4/80^{+}$ macrophages (C), $Ly\text{-}6G^{-}/F4/80^{-}$ monocytes (D), and $Ly\text{-}6G^{+}/CD19^{-}$ granulocytes (G) were quantified. Total monocytes were separated into $Ly\text{-}6C^{hi}$ (E) and $Ly\text{-}6C^{lo}$ (F) populations. Of lymphoid cells, $CD3^{+}/CD19^{-}$ T cells (H) and $CD3^{-}/CD19^{+}$ B cells (I) were quantified. Fold change is relative to the average number of cells among male My-MR-intact mice in each independent experiment. Statistical significance was determined by two-way ANOVA with Bonferroni's post-test. ns = not significant. (n=11)

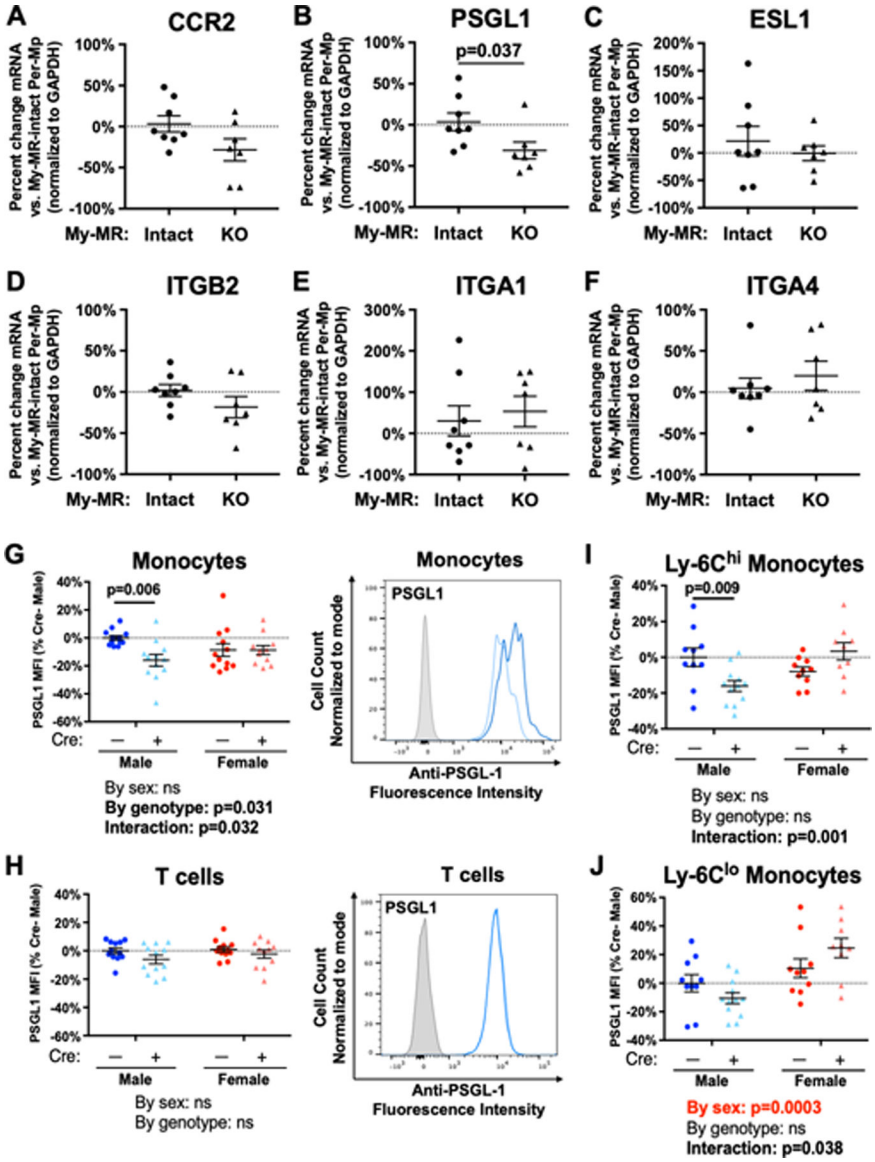

Figure 4. PSGL1 expression in monocytes and macrophages is attenuated with My-MR-KO.

(A-F) Quantification of mediators of leukocyte trafficking: C-C chemokine receptor type 2 (CCR2) (A), P-selectin glycoprotein ligand-1 (PSGL1) (B), E-selectin ligand (ESL1) (C), integrin beta-2 (ITGB2) (D), integrin alpha-1 (ITGA1) (E), and integrin alpha-4 (ITGA4) (F) mNRA normalized to GAPDH expression in resident Per-Mp from ApoE-KO/My-MR-intact (circles, Cre^{-}) and ApoE-KO/My-MR-KO (triangles, Cre^{+}) mice. Fold change is relative to average gene expression from My-MR-intact mice in each independent experiment. Statistical significance was determined by two-tailed Student's t test. *$p<0.05$. (n=8) (G-H) Surface protein expression of PSGL1 was quantified by the median fluorescence intensity (MFI) of anti-PSGL1 staining on circulating $CD11b^{+}/Ly\text{-}6G^{-}/CD115^{+}$ monocytes (G) and $CD3^{+}/CD11b^{-}$ T cells (H) from male and female My-MR-intact (circles, Cre^{-}) and My-MR-KO (triangles, Cre^{+}) mice at baseline. Representative histograms depict anti-PSGL1 fluorescence intensity from male My-MR-intact (dark blue) and My-MR-

KO (light blue) mice. Isotype control shown in gray. (I-J) MFI of anti-PSGL1 staining on circulating Ly-6C^{hi} (I) and Ly-6C^{lo} (J) monocytes as in G-H. Data in G-J is represented as the percentage change relative to the average MFI among male My-MR-intact mice (Cre^{-} males) in each independent experiment. Statistical significance was determined by two-way ANOVA with Bonferroni's post-test. (n=11–12)

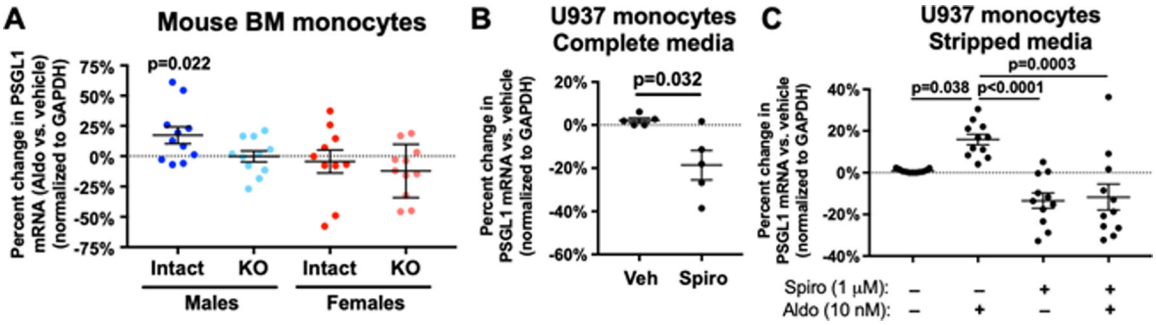

Figure 5. Aldosterone regulates monocyte PSGL1 mRNA in a MR-dependent manner in male mouse and human monocytes.

(A) PSGL1 mRNA expression quantified in bone marrow-derived (BM) monocytes from male and female My-MR-intact and My-MR-KO mice treated with 10 nM aldosterone (Aldo) or vehicle (Veh) for 6 hours (n=10–11). (B) PSGL1 mRNA expression quantified by RT-PCR in U937 cells cultured in complete media and treated with 1 μM spironolactone (Spiro) or Veh for 6 hours (n=5). (C) PSGL1 mRNA expression quantified by RT-PCR in U937 cells cultured in charcoal-stripped media and treated with 1 μM Spiro and/or 10 nM Aldo (n=11). Data is represented as percent change in PSGL1 mRNA in Aldo- and/or Spiro-treated cells vs. Veh for each group. Statistical significance was determined in (A) by one-way ANOVA with Bonferroni's post-test, in (B) by two-tailed Mann-Whitney test, and in (C) by Kruskal-Wallis test with Dunn's post-test.

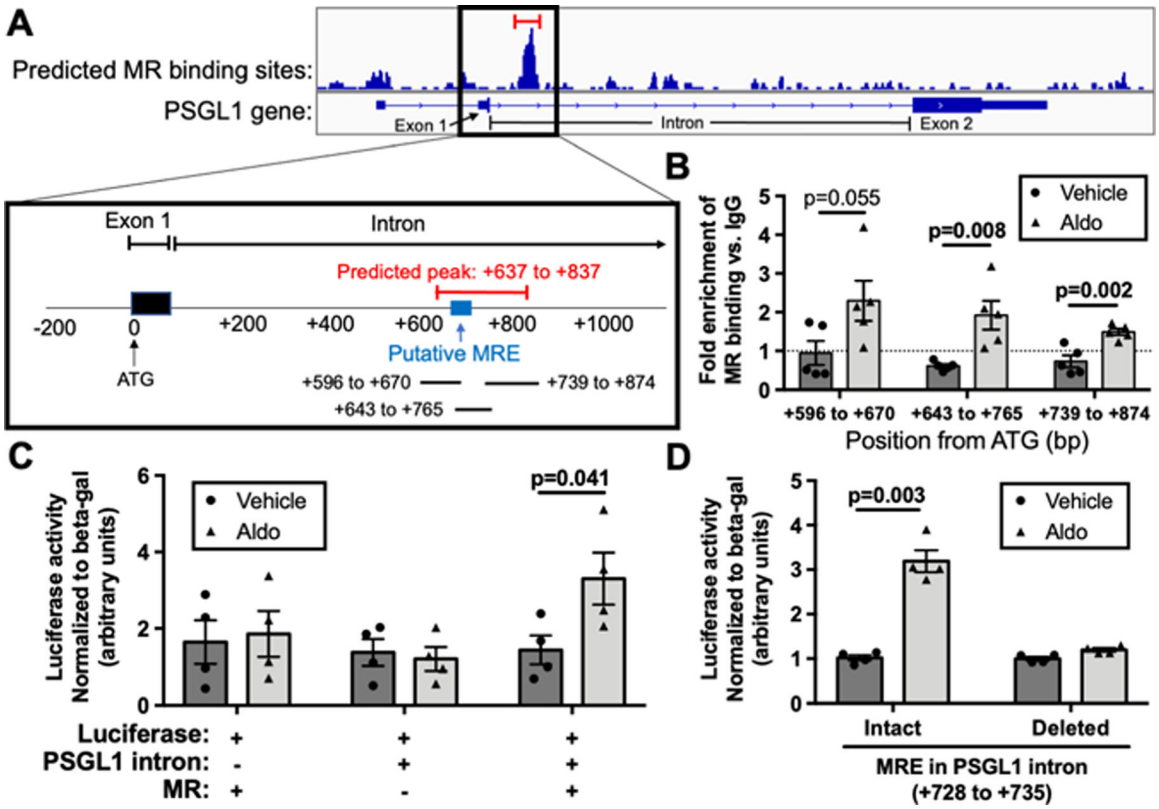

Figure 6. Myeloid MR regulates PSGL1 transcription in response to aldosterone by binding to a MR response element in intron 1 of the PSGL1 gene.

(A) The PSGL1 gene is shown with visualization of glucocorticoid receptor binding from publicly available chromatin immunoprecipitation (ChIP)-sequencing data (top). A putative MR response element (MRE) from *in silico* analysis of transcription factor binding sites within the PSGL1 intron is shown (bottom). (B) MR binding to a predicted MRE in the single intron of the PSGL1 gene was assessed by ChIP-qPCR using the primers listed on the bottom of Figure (A) in human U937 monocytes treated with 10 nM aldosterone (Aldo, triangles) or vehicle (circles). Fold enrichment of MR is expressed for anti-MR antibody binding to immunoprecipitated chromatin relative to the IgG isotype control for each treatment. (n=5) (C) Luciferase reporter activity in HEK293 cells co-transfected with a luciferase-reporter plasmid regulated by the PSGL1 intronic sequence +/− a plasmid expressing human MR and treated with 10 nM Aldo or vehicle. (D) Luciferase activity in HEK293 cells co-transfected with human MR and luciferase-reporter plasmids including the PSGL1 intronic sequence with an intact or deleted MRE, and treated with 10 nM Aldo or vehicle. (n=4) Statistical significance in B-D by two-tailed Mann-Whitney test.