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G2A Deficiency in Mice Promotes Macrophage Activation and Atherosclerosis

David T. Bolick, Marcus D. Skaflen, Laura E. Johnson, Seong-Chun Kwon, Deborah Howatt, Alan Daugherty, Kodi S. Ravichandran, Catherine C. Hedrick

Abstract—G2A is a stress-inducible G protein-coupled receptor that is expressed on several cell types within atherosclerotic lesions. We demonstrated previously that G2A deficiency in mice increased aortic monocyte recruitment and increased monocyte:endothelial interactions. To investigate the impact of G2A deficiency in macrophages, we isolated peritoneal macrophages from G2A^{+/+}ApoE^{-/-} and G2A^{-/-}ApoE^{-/-} mice. G2A^{-/-}ApoE^{-/-} macrophages had significantly lower apoptosis than control macrophages. The prosurvival genes BCL-2, BCL-xL, and cFLIP were increased in G2A^{-/-}ApoE^{-/-} macrophages. Macrophages from G2A^{-/-}ApoE^{-/-} mice also had increased proinflammatory status that was indicative of a M1 macrophage phenotype. This was indicated by significantly increased nuclear translocation of nuclear factor κ B, as well as production of interleukin-12p40, tumor necrosis factor α , and interleukin-6, and reduced expression of arginase-I. Moreover, G2A^{-/-}ApoE^{-/-} macrophages had reduced ability to engulf apoptotic cells in vitro. We examined atherosclerosis in mice fed a Western diet for 10 weeks and found that G2A deficiency increased lesion size in the aortic root by 50%. Plasma lipid levels were not changed in G2A^{-/-}ApoE^{-/-} mice. However, we found that absence of G2A increased the number of aortic macrophages and attenuated apoptosis in this cell type. Moreover, bone marrow transplantation studies indicated that deficiency of G2A in marrow-derived cells significantly contributed to atherosclerosis development. In the absence of G2A, increased macrophage activation and decreased apoptosis is associated with accumulation of macrophages in the aorta and increased atherosclerosis. (*Circ Res.* 2009;104:318-327.)

Key Words: apoptosis ■ macrophages ■ vascular inflammation ■ atherosclerosis

Macrophages and T lymphocytes play critical roles in the initiation and development of atherosclerosis.¹ Activated endothelium recruits monocytes by secretion of chemoattractants, after which monocytes bind to and subsequently transmigrate through the endothelial layer.²⁻⁶ In addition to macrophages, CD4⁺ lymphocytes are detected in early atherogenesis and late-stage unstable atherosclerotic lesions, consistent with a role for acquired immunity in lesion development.⁷⁻⁹ Lesional lymphocytes secrete the inflammatory cytokine interferon- γ , which further activate macrophages and vascular cells.¹⁰⁻¹³

The G protein-coupled receptor G2A is a stress-inducible receptor. Overexpression in fibroblasts causes cell cycle arrest at the G2 phase of mitosis, thus the name G2A for G2 Accumulation.¹⁴ G2A expression attenuates Bcr-Abl oncogene-mediated cell proliferation, whereas mice lacking G2A have an increased mortality rate in an

oncogene-induced model of leukemia.¹⁵ The endogenous ligand for G2A is unknown, although putative ligands include lysophosphatidylcholine,^{16,17} 9(S)HODE,¹⁸ and possibly other free fatty acids.¹⁸ G2A and other receptors within the OGR1 family (TDAG8, GPR4, and OGR1) respond to changes in extracellular pH.¹⁹ However G2A is less responsive to pH changes compared to other receptor family members.²⁰

G2A is highly expressed on macrophages and lymphocytes, with lower expression found on macrovascular endothelium.^{21,22} G2A has been localized to atherosclerotic lesions in mice, consistent with a contributory role in the disease process.²³ We have reported that G2A deficiency increases monocyte:endothelial interactions in vivo, resulting in increased monocyte accumulation in aorta.²² In this prior study, we demonstrated a critical role of G2A in endothelium; however, the impact of G2A deficiency on the macrophage was not investigated.

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In the present study, we hypothesize that G2A deficiency in macrophages would result in a proinflammatory macrophage phenotype. Our data are consistent with this hypothesis, because macrophages from G2A^{-/-}ApoE^{-/-} show increased cytokine secretion, nuclear factor (NF)κB activation, and associated increases in survival gene expression. G2A^{-/-}ApoE^{-/-} mice fed a diet high in saturated fat for 10 weeks develop increased aortic root atherosclerosis compared to G2A^{+/+}ApoE^{-/-}. These data demonstrate that G2A deficiency results in a proinflammatory macrophage M1 phenotype that is associated with increased atherosclerosis.

Materials and Methods

Detailed methods can be found in the online data supplement at <http://circres.ahajournals.org>. For the present studies, we fed G2A^{+/+}ApoE^{-/-} and G2A^{-/-}ApoE^{-/-} double knockout mice a Western (Harlan Teklad 88137) for 10 weeks. We measured atherosclerosis using aortic root and en face techniques. In some studies, thioglycollate-elicited peritoneal macrophages were obtained from mice for measurements of apoptosis using flow cytometry and real-time PCR and macrophage inflammatory phenotype using ELISA and real-time PCR. We also performed atherosclerosis measurements in ApoE^{-/-} and Ldlr^{-/-} recipient mice fed a Western diet for 10 weeks that had received ApoE^{-/-}, G2A^{-/-}ApoE^{-/-}, Ldlr^{-/-}, or G2A^{-/-}Ldlr^{-/-} bone marrow cells in a series of bone marrow transplantation studies.

Results

Activation of NFκB in G2A-Deficient Macrophages

On activation, NFκB is translocated to the nucleus where it acts as a transcription factor. We found that NFκB p65 levels were significantly increased in macrophage nuclear extracts from G2A^{-/-}ApoE^{-/-} mice (Figure 1A). Phosphorylation of nuclear NFκB p65, another measure of NFκB activation, was also increased in G2A^{-/-}ApoE^{-/-} macrophages (data not shown). AKT is involved in NFκB signaling,²⁴ and AKT phosphorylation activates the BCL family of antiapoptotic factors. Peritoneal macrophages from G2A^{-/-}ApoE^{-/-} mice showed increased AKT phosphorylation compared to G2A^{+/+}ApoE^{-/-} control (Figure 1B). Figure 1C represents densitometry of 6 mice per group.

G2A^{-/-}ApoE^{-/-} Macrophages Have Reduced Apoptosis

We examined expression of genes involved in inflammation and apoptosis. Several survival gene targets of NFκB were upregulated in G2A^{-/-}ApoE^{-/-} macrophages compared to control, including BCL-2, BCL-xL, and cFLIP (Figure 2A). In addition, IAP2 was increased 1.8-fold, and expression of p73 and p53, 2 proapoptotic genes, was reduced 2.7-fold (Figure 2A). Interestingly though, we observed significant upregulation of several other proapoptotic genes, including FasL, PAK7, and caspase-12 (Figure 2A), suggesting that G2A expression in the macrophage must regulate apoptotic/survival pathways, and in the absence of G2A, these pathways become dysregulated.

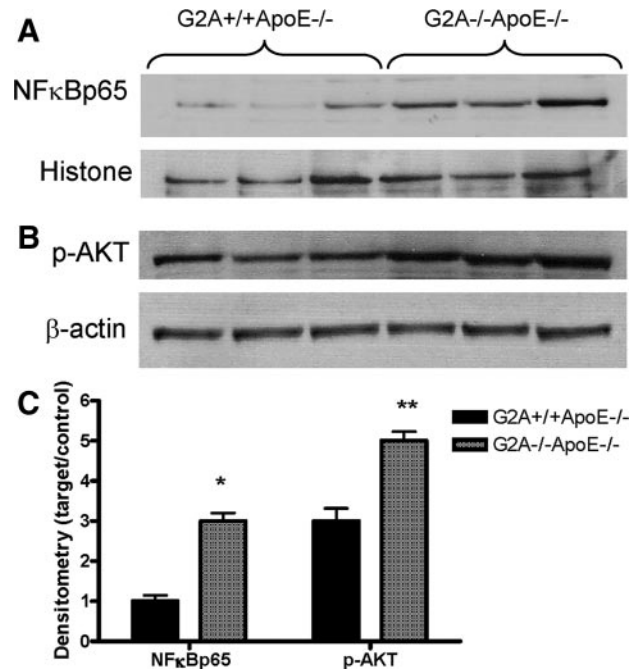


Figure 1. NFκB is activated in G2A^{-/-} macrophages. Cytosol and nuclear extracts were isolated as described in Materials and Methods from G2A^{+/+}ApoE^{-/-} and G2A^{-/-}ApoE^{-/-} mice fed a Western diet for 10 weeks. The images shown are peritoneal macrophages isolated from 3 mice from each group. A, Nuclear proteins (50 μg) were analyzed by SDS-PAGE for NFκBp65 or histone. B, Cytosolic proteins (50 μg) were used to analyze levels of phosphorylated AKT and β-actin. C, Graph represents densitometry of 6 mice per group. **P*<0.002, significantly greater than G2A^{+/+}ApoE^{-/-} control; ***P*<0.005, significantly greater than control.

To examine whether the macrophages exhibited a functionally proapoptotic or prosurvival phenotype, we performed several assays. First, we measured Annexin V staining on freshly isolated peritoneal macrophages from G2A^{-/-}ApoE^{-/-} and G2A^{+/+}ApoE^{-/-} mice using flow cytometry (Figure 2B). G2A^{-/-}ApoE^{-/-} macrophages showed significantly less Annexin V staining, as measured by flow cytometry (Figure 2B). Alternatively, peritoneal macrophages were plated overnight on chamber slides and stained for TUNEL or cleaved caspase-3. G2A^{-/-}ApoE^{-/-} macrophages showed significantly less TUNEL staining, (*P*<0.001; Figure 2C), and less cleaved caspase-3 than G2A^{+/+}ApoE^{-/-} (Figure 2C). Taken together, these results indicate that G2A deficiency in macrophages activates prosurvival signaling pathways thereby preventing apoptosis, even though several proapoptotic pathways are activated in these macrophages.

G2A^{-/-}ApoE^{-/-} Macrophages Are Proinflammatory and Have Impaired Apoptotic Cell Engulfment

We next examined the inflammatory phenotype of G2A^{-/-}ApoE^{-/-} and G2A^{+/+}ApoE^{-/-} macrophages. IL-12p70, tumor necrosis factor (TNF)α, monocyte chemoattractant protein (MCP)-1, IL-10, and IL-6 secretion into culture media

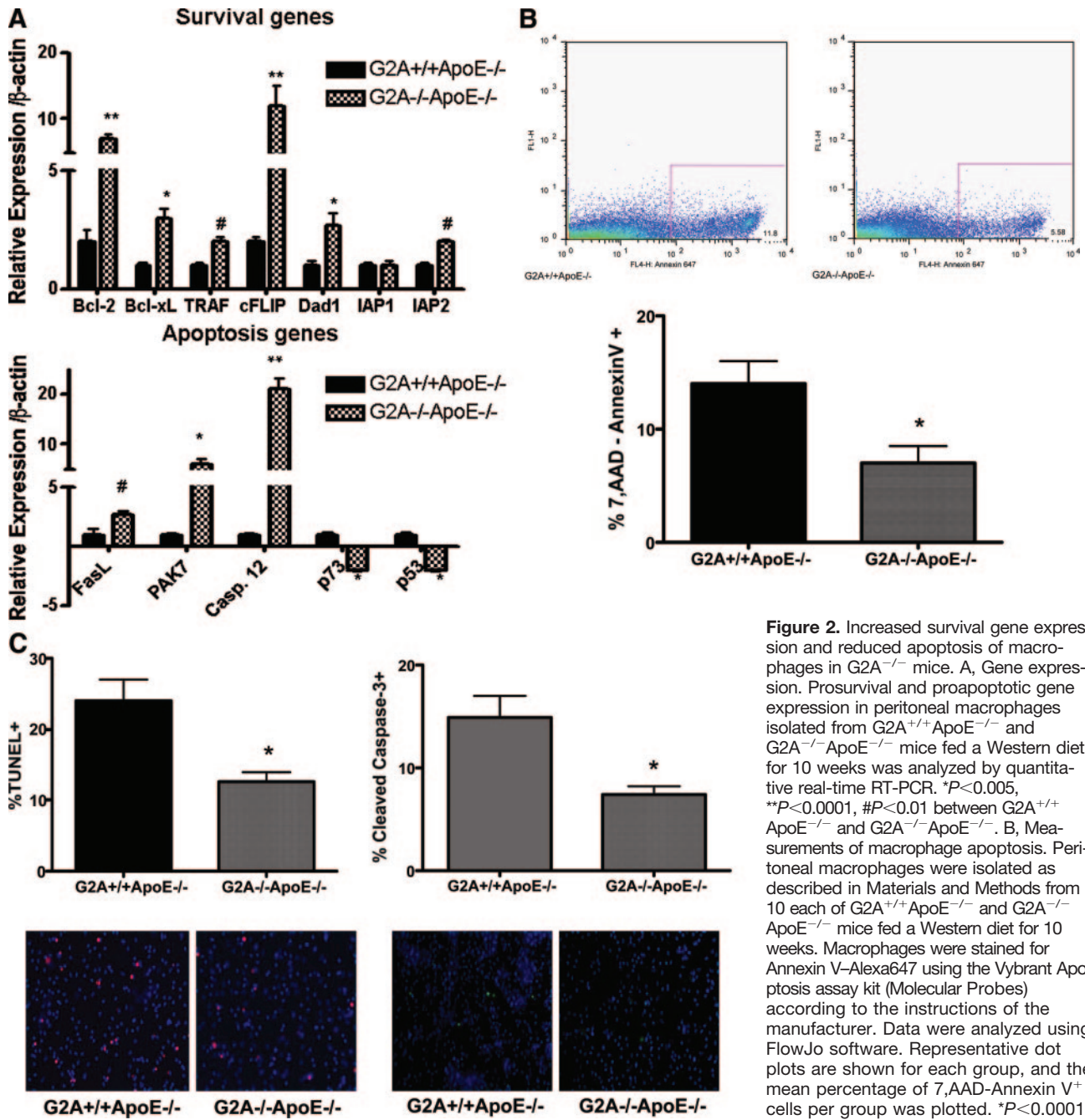


Figure 2. Increased survival gene expression and reduced apoptosis of macrophages in G2A^{-/-} mice. **A**, Gene expression. Prosurvival and proapoptotic gene expression in peritoneal macrophages isolated from G2A^{+/+}ApoE^{-/-} and G2A^{-/-}ApoE^{-/-} mice fed a Western diet for 10 weeks was analyzed by quantitative real-time RT-PCR. **P*<0.005, ***P*<0.0001, #*P*<0.01 between G2A^{+/+}ApoE^{-/-} and G2A^{-/-}ApoE^{-/-}. **B**, Measurements of macrophage apoptosis. Peritoneal macrophages were isolated as described in Materials and Methods from 10 each of G2A^{+/+}ApoE^{-/-} and G2A^{-/-}ApoE^{-/-} mice fed a Western diet for 10 weeks. Macrophages were stained for Annexin V–Alexa647 using the Vybrant Apoptosis assay kit (Molecular Probes) according to the instructions of the manufacturer. Data were analyzed using FlowJo software. Representative dot plots are shown for each group, and the mean percentage of 7-AAD-Annexin V⁺ cells per group was plotted. **P*<0.0001, significantly less than G2A^{+/+}ApoE^{-/-} control. **C**, TUNEL and caspase-3 stain-

ing. Peritoneal macrophages from each group were stained for TUNEL using the tetramethylrhodamine red in situ TUNEL assay kit (Roche Applied Sciences) or stained for cleaved caspase-3 as described in Materials and Methods. Nuclei were stained with DAPI (blue). **P*<0.0005, significantly less than G2A^{+/+}ApoE^{-/-} control for TUNEL; **P*<0.002, significantly less than G2A^{+/+}ApoE^{-/-} control for cleaved caspase-3.

were all significantly higher in G2A^{-/-}ApoE^{-/-} macrophages compared to control (Figure 3A). Interferon- γ levels were unchanged. Additionally, quantitative real-time RT-PCR was performed on RNA isolated from peritoneal macrophages from G2A^{-/-}ApoE^{-/-} and G2A^{+/+}ApoE^{-/-} mice, and we observed similar inductions of cytokine mRNA expression (Figure 3B). In addition, we found significant reductions in Arginase-I and transform-

ing growth factor β expression in G2A^{-/-}ApoE^{-/-} macrophages (Figure 3B), both of which have been linked to a M2 alternative, antiinflammatory macrophage phenotype.²⁵ We also found similar changes in gene expression in macrophages isolated from whole aorta (data not shown). Taken together, these data suggest that G2A deficiency promotes an inflammatory M1-like macrophage phenotype.

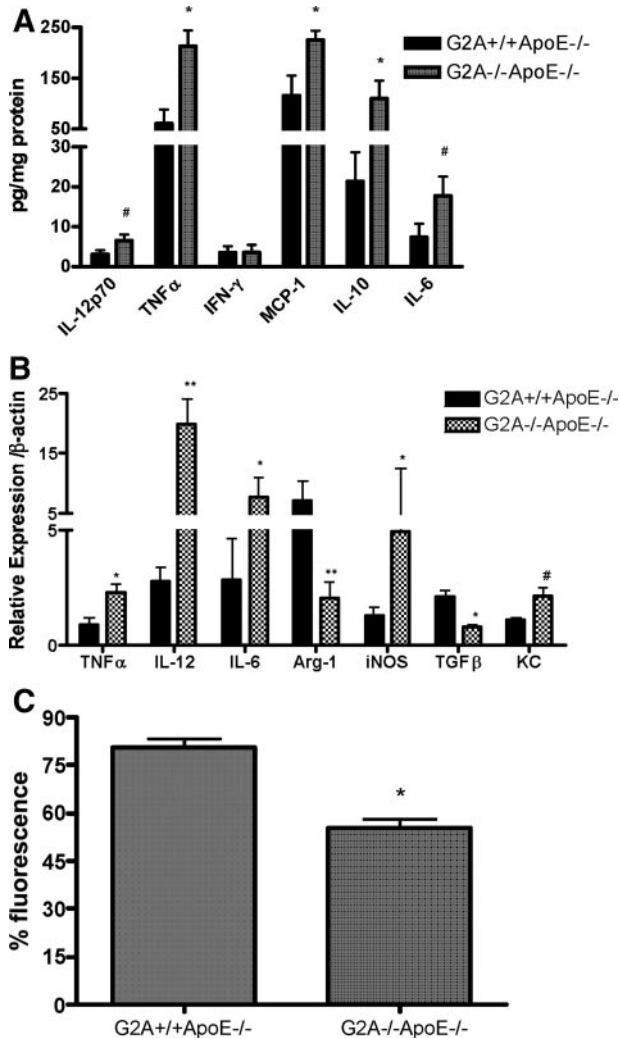


Figure 3. Inflammatory cytokine production by G2A^{-/-} macrophages. A, Cytometric bead arrays were used to analyze inflammatory cytokine secretion from G2A^{+/+}ApoE^{-/-} and G2A^{-/-}ApoE^{-/-} peritoneal macrophages fed a Western diet for 10 weeks. Assays were conducted according to the instructions of the manufacturer. [#]*P*<0.02, significantly greater than G2A^{+/+}ApoE^{-/-} control; ^{*}*P*<0.005, significantly greater than G2A^{+/+}ApoE^{-/-}. B, Quantitative real-time RT-PCR of macrophage inflammatory markers represented as relative expression and normalized to β-actin. ^{*}*P*<0.001, ^{**}*P*<0.0001, [#]*P*<0.008. C, Peritoneal macrophages from G2A^{+/+}ApoE^{-/-} and G2A^{-/-}ApoE^{-/-} mice fed a Western diet for 10 weeks were incubated with fluorescently labeled apoptotic Jurkat cells for 15 minutes for an engulfment assay. The percentage of engulfed cells was determined as a measure of fluorescence by flow cytometry. ^{*}*P*<0.03. Data represent n=4 mice per group.

Next, we measured the ability of the G2A^{-/-}ApoE^{-/-} macrophages to engulf apoptotic cells. Efficient clearance of apoptotic cells by phagocytic macrophages is associated with promotion of an antiinflammatory, M2-like phenotype. As shown in Figure 3C, G2A^{-/-}ApoE^{-/-} macrophages showed reduced ability to engulf apoptotic cells in vitro. Because defective apoptotic cell clearance from the vessel wall is associated with increased atherosclerosis,^{26,27} these data would suggest that G2A deficiency in macrophages would contribute to atherosclerosis development.

Apoptosis and Antiapoptotic Gene Expression After Treatment With Oxidized Low-Density Lipoprotein

Peritoneal macrophages from 6 each of G2A^{+/+}ApoE^{-/-} and G2A^{-/-}ApoE^{-/-} mice fed chow were isolated and stimulated with the treatment of with oxidized low-density lipoprotein (LDL) (50 μg/mL) for 18 hours. After treatment, cells were collected for measurement of apoptosis and survival gene expression. Apoptosis, as measured by Annexin V staining, was significantly reduced in G2A^{-/-}ApoE^{-/-} macrophages compared to G2A^{+/+}ApoE^{-/-} control (*P*<0.002). Treatment with oxLDL increased apoptosis in G2A^{+/+}ApoE^{-/-} by 50% (*P*<0.01) but did not significantly increase apoptosis in G2A^{-/-}ApoE^{-/-} macrophages. Figure 4A shows representative dot plots from each group. The survival genes BCL-2, BCL-xL, and cFLIP, as well as inducible NOS expression, were all significantly increased in G2A^{-/-}ApoE^{-/-} macrophages compared to control (*P*<0.001), whereas treatment with oxLDL further increased survival gene and inducible NOS expression in G2A^{-/-}ApoE^{-/-} macrophages (*P*<0.002) (Figure 4B). Treatment of G2A^{+/+}ApoE^{-/-} macrophages with oxLDL reduced expression of the survival genes BCL-2, BCL-xL, and cFLIP (*P*<0.005). Arginase I expression was significantly reduced in untreated G2A^{-/-}ApoE^{-/-} compared to G2A^{+/+}ApoE^{-/-} (*P*<0.02) but was restored to control levels on stimulation with oxLDL (Figure 4B).

Atherosclerosis Lesion Analysis and Characterization

We next examined atherosclerosis development in G2A-deficient mice after 10 weeks of Western diet feeding. We found that fasting total cholesterol concentrations were elevated in both groups. There were no significant differences between G2A^{+/+}ApoE^{-/-} and G2A^{-/-}ApoE^{-/-} mice in blood glucose levels (306±36.2 versus 282.7±48.1 mg/dL), total cholesterol (1084±107.4 versus 972.5±161.5 mg/dL), high-density lipoprotein (HDL) (16.1±2.1 versus 16.4±3.1 mg/dL), LDL (1052±124.9 versus 943.3±157 mg/dL), or triglycerides (128.7±28.6 versus 132.4±28.9 mg/dL), respectively. Body weight at the end of the experiment also did not differ between groups (22.2 g±2.8 g for G2A^{+/+}ApoE^{-/-} vs 21.6±3.6 g for G2A^{-/-}ApoE^{-/-}).

G2A deficiency did lead to significantly increased concentrations of MCP-1 and IL-6 in plasma, however (*P*<0.005; Figure 5). Plasma concentrations of interferon-γ, IL-12p70, IL-10, and TNFα were below the detection sensitivity of the assay in all groups (data not shown).

Aortic root sections from G2A^{+/+}ApoE^{-/-} and G2A^{-/-}ApoE^{-/-} mice were collected, and lesion area was measured in both the sinus and the ascending aortas as indicated in Figure 6A, with the “0” point indicating the transition between these areas. After 10 weeks of Western diet feeding, G2A^{-/-}ApoE^{-/-} mice showed increased atherosclerosis development within -240 to +240 μm from the transition

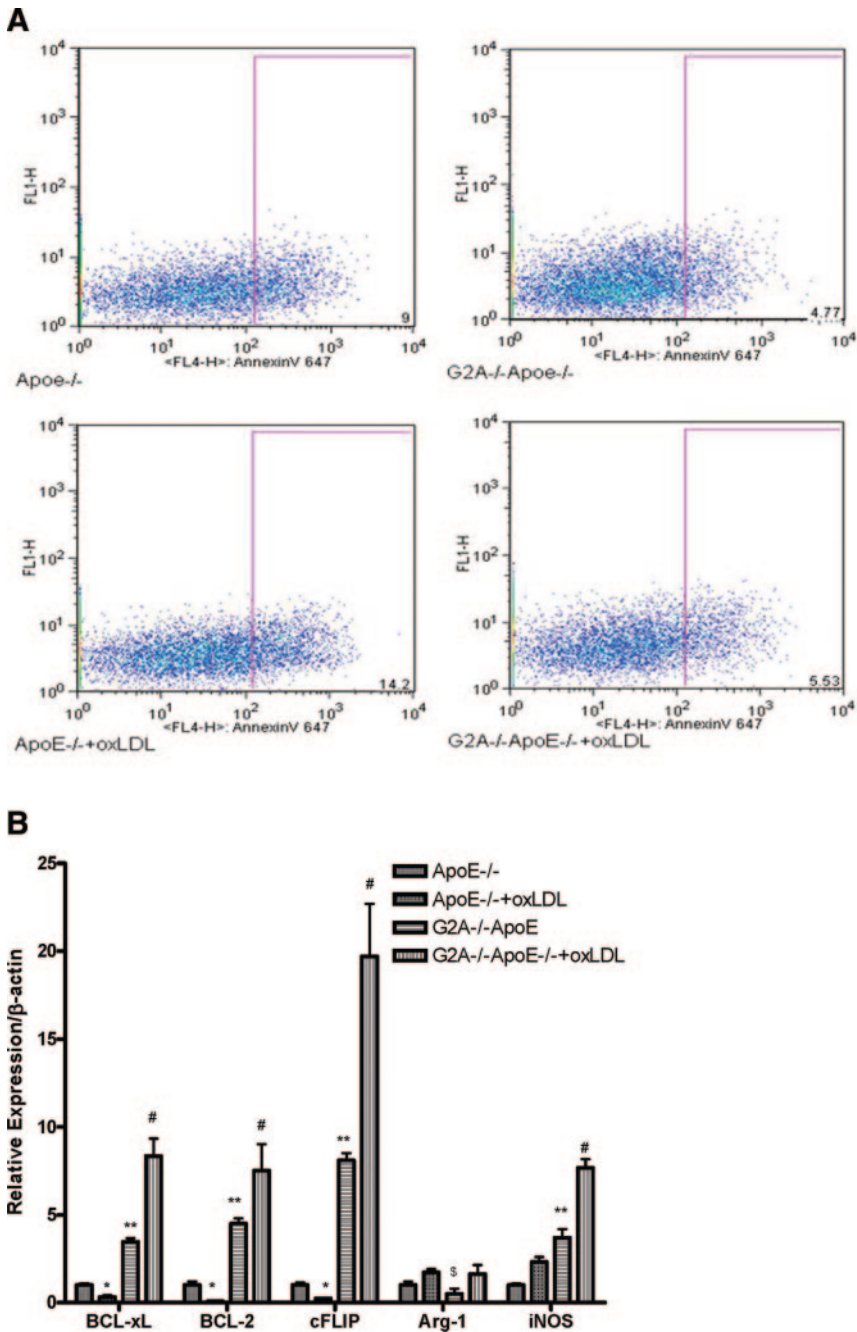


Figure 4. oxLDL treatment of peritoneal macrophages. A, Peritoneal macrophages were isolated from 6 mice per group fed chow, treated with or without oxLDL (50 μ g/mL) for 18 hours and stained for Annexin V–Alexa647 using the Vybrant Apoptosis assay kit (Molecular Probes) according to the instructions of the manufacturer. Data were analyzed using FlowJo software. Representative dot plots are shown for each group. B, Quantitative real-time RT-PCR of antiapoptosis genes and macrophage inflammatory markers represented as relative expression and normalized to β -actin. * $P < 0.005$, significantly lower than $G2A^{+/+}ApoE^{-/-}$ control; ** $P < 0.001$, greater than $G2A^{+/+}ApoE^{-/-}$ control; # $P < 0.002$, greater than $G2A^{-/-}ApoE^{-/-}$; \$ $P < 0.02$, less than $G2A^{+/+}ApoE^{-/-}$ control.

($P < 0.001$; Figure 6A). Movat pentachrome and picrosirius red staining of 5- μ m sections from aortic roots (taken at +160 μ m from the transition) demonstrated that $G2A$ deficiency led to greater collagen content in the aortic root, suggesting formation of complex atherosclerotic plaques (Figure 6B). Using Movat stain, collagen content is apparent by dark purple to black coloration.²⁸ Using picrosirius red staining, collagen content of the lesion is visible by red staining under normal light. Under polarized light, red, orange, yellow, and green colors are apparent (the colors of collagen fibers in order of decreasing thickness) (Figure 6C).²⁹

Absence of $G2A$ in Marrow-Derived Cells Contributes to Early Atherosclerosis Development

Bone marrow transplantation studies were performed in $ApoE^{-/-}$ recipients using $ApoE^{-/-}$ and $G2A^{-/-}ApoE^{-/-}$ bone marrow and in $Ldlr^{-/-}$ recipients using $Ldlr^{-/-}$ and $G2A^{-/-}Ldlr^{-/-}$ bone marrow. After 6 weeks of reconstitution, mice were placed on a Western diet for 10 weeks. Atherosclerosis was measured using en face analysis. First, as expected, the percentage plaque area was greater in $ApoE^{-/-}$ recipients receiving $apoE^{-/-}$ marrow compared to $Ldlr^{-/-}$ recipients receiving $Ldlr^{-/-}$ marrow (Figure 7). In the $apoE^{-/-}$ recipients, we observed a trend toward increased

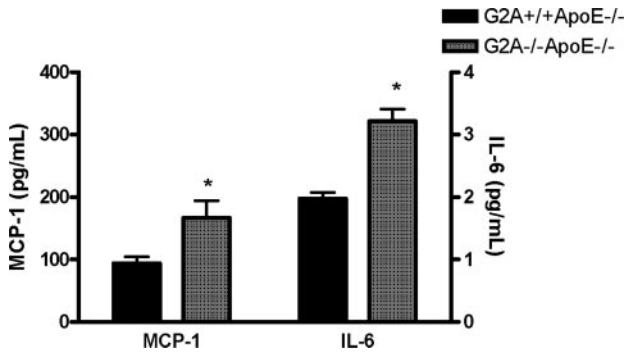


Figure 5. Plasma cytokine concentrations. Blood was collected from 15 each of G2A^{+/+}ApoE^{-/-} and G2A^{-/-}ApoE^{-/-} mice after 10 weeks on Western diet via cardiac puncture. Plasma was analyzed for cytokine production using a cytometric bead array and flow cytometry. **P*<0.002, significantly higher than G2A^{+/+}ApoE^{-/-} control.

plaque area in the mice that received G2A^{-/-}ApoE^{-/-} bone marrow, but the data did not reach statistical significance. However, in the Ldlr^{-/-} recipients, absence of G2A in marrow-derived cells significantly increased aortic plaque area, *P*<0.003 (Figure 7). Taken together, these data suggest that G2A deficiency in macrophages contributes significantly to atherosclerosis development yet does

not rule out some contribution of G2A deficiency in non-marrow-derived cells.

Higher Macrophage Content in Aortic Wall of G2A^{-/-} Mice

Finally, we examined macrophage numbers in the aortic wall using a novel flow cytometric method developed by Ley and colleagues.³⁰ Macrophage content in vivo in the aortic wall of G2A^{-/-}ApoE^{-/-} mice was significantly higher than that of ApoE^{-/-} control (*P*<0.002), as measured by dual staining for CD45 and F4/80 (Figure 8A). However, total monocyte and lymphocyte counts in blood were similar between the 2 experimental groups (data not shown). Thus, the increase in aortic wall macrophage accumulation does not occur as a result of increased leukocyte numbers in blood of the G2A^{-/-}ApoE^{-/-} mice. We anticipate that the increased aortic wall content is attributable to increased monocyte:endothelial cell interactions in the arterial wall in the absence of G2A, which we have previously reported,²² as well as increased macrophage survival. Indeed, G2A^{-/-}ApoE^{-/-} aortic root cross-sections, whereas having a greater plaque area and macrophage content had significantly less TUNEL⁺/DAPI⁺ costaining (Figure 8B). The regions of positive TUNEL staining in G2A^{-/-}ApoE^{-/-} lesions showed very little costaining for either DAPI nuclei or MAC-2 macrophage,

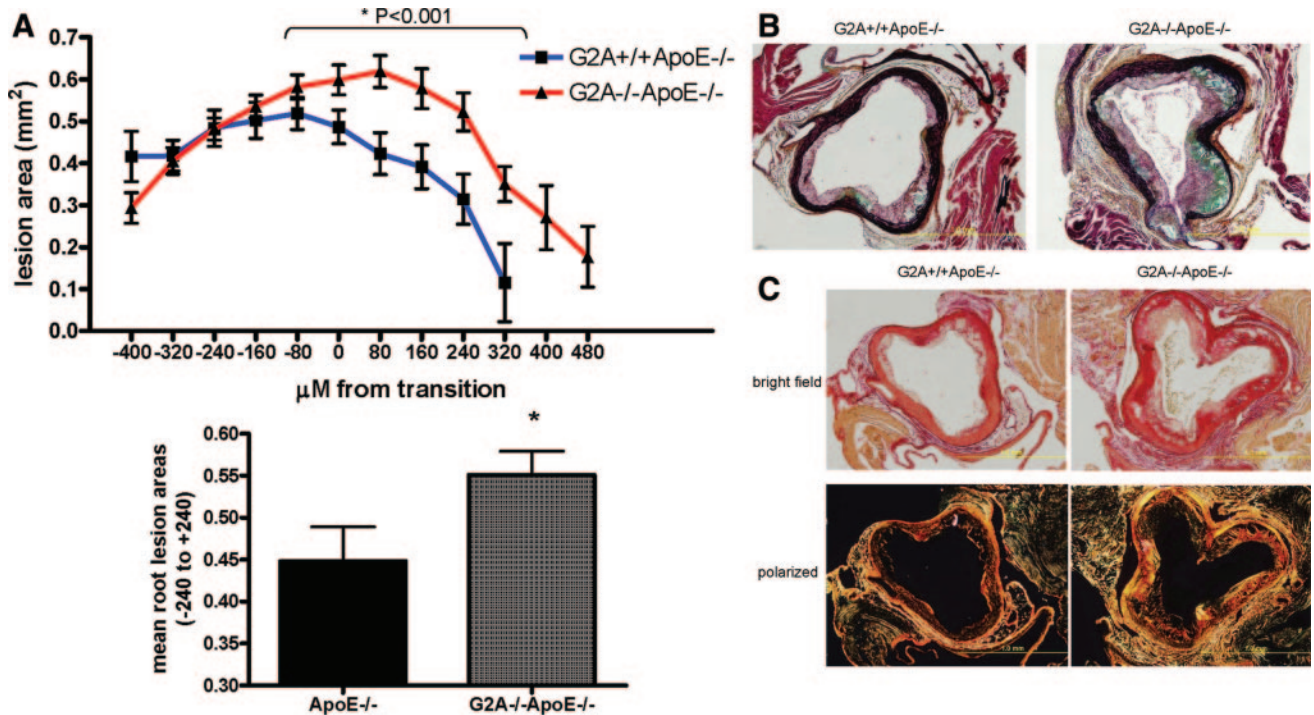


Figure 6. G2A^{-/-}ApoE^{-/-} mice have increased atherosclerosis after 10 weeks of diet feeding. A, Atherosclerotic lesion analysis. Aortic roots from G2A^{+/+}ApoE^{-/-} and G2A^{-/-}ApoE^{-/-} mice were embedded in paraffin. Five-micron sequential sections from the aortic sinus and the descending aorta (-400 to +480 μm from the transition) were sectioned and stained using oil red O, and lesion area was quantified using Image Pro software analysis. **P*<0.001, significantly greater lesion area than G2A^{+/+}ApoE^{-/-} control (from -240 to +240 μm). B, Movat staining of aortic root sections. Five-micron sections from the aortic sinus and the descending aorta at +160 μm were stained using pentachrome Movat stain. Collagen and reticulum fibers are yellow to greenish yellow. C, Picrosirius red staining of aortic root sections. Five-micron sections at +160 μm from the transition were stained using picrosirius red stain and visualized under bright field and polarized light. Under normal light, collagen content of the lesion is visible by red staining. Under polarized light, red, orange, yellow, and green colors are apparent (the colors of collagen fibers in order of decreasing thickness).²⁹

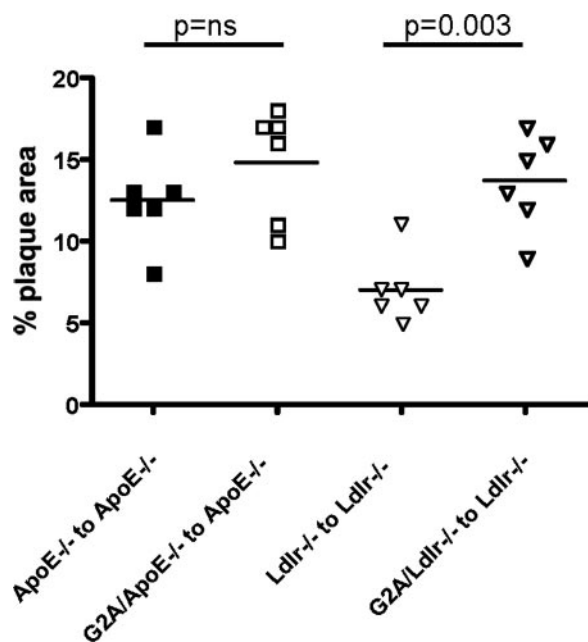


Figure 7. Absence of G2A in marrow-derived cells contributes to atherosclerosis. Female LDLR^{-/-} or apoE^{-/-} mice were irradiated and transplanted with donor bone marrow (Ldlr^{-/-}, G2A^{-/-}Ldlr^{-/-}, apoE^{-/-}, G2A^{-/-}ApoE^{-/-}) as indicated in the figure. Mice were reconstituted for 6 weeks, after which they were fed a Western diet for 10 weeks. Atherosclerosis was assessed by measuring en face aortic lesion area.

indicating these areas are necrotic. We found an $\approx 5\%$ increase in the percentage necrotic core/total plaque area ratio in the aortic roots of G2A^{-/-}ApoE^{-/-} mice compared to G2A^{+/+}ApoE^{-/-} mice. Aortic root sections from ApoE^{-/-} mice had more macrophage staining, as measured by MAC-2⁺ staining, as well as greater TUNEL⁺/DAPI⁺ staining. These data combined with our in vitro data on macro-

phage function suggest that the reduced macrophage apoptosis observed in G2A^{-/-}ApoE^{-/-} mice contributes to aortic macrophage accumulation and atherosclerosis.

Discussion

G2A is a G protein-coupled receptor that is highly expressed in macrophages and lymphocytes.³¹ The endogenous ligand for G2A remains unclear^{16,18,32,33}; however, there is some evidence that G2A is involved in atherosclerotic lesion development in animal models.^{21,23} We have recently found that absence of G2A in mice contributes to cholesterol gallstone disease,³⁴ although G2A has not, to date, been defined as a *Lith* gene. The gene in humans that corresponds to G2A is GPR132. Currently, there are no known reported associations of polymorphisms in GPR132 with clinical disease in humans. Recently, however, we have discovered single-nucleotide polymorphisms within GPR132 that are associated with internal intimal media thickness of the carotid artery in patients (data not shown). Thus, GPR132 (G2A) may indeed represent a clinically relevant gene for lipid metabolism and atherosclerosis.

In the present study, we investigated the impact of G2A deficiency on macrophage function and atherosclerosis in ApoE^{-/-} mice. G2A^{-/-}ApoE^{-/-} mice fed a fat-enriched diet for 10 weeks had significantly greater aortic sinus lesion area compared to G2A^{+/+}ApoE^{-/-} mice. G2A^{-/-}ApoE^{-/-} mice additionally had significantly higher numbers of macrophages present in the aortic wall compared to G2A^{+/+}ApoE^{-/-}. Although there was no difference in circulating lipid levels, G2A^{-/-}ApoE^{-/-} mice had significantly greater plasma IL-6 and MCP-1 levels. Moreover, macrophages isolated from these mice had increased production of proinflammatory cytokines and reduced expression of antiinflammatory genes. These data suggest that G2A deficiency promotes a proinflammatory M1 macrophage phenotype that contributes to atherosclerotic lesion development.

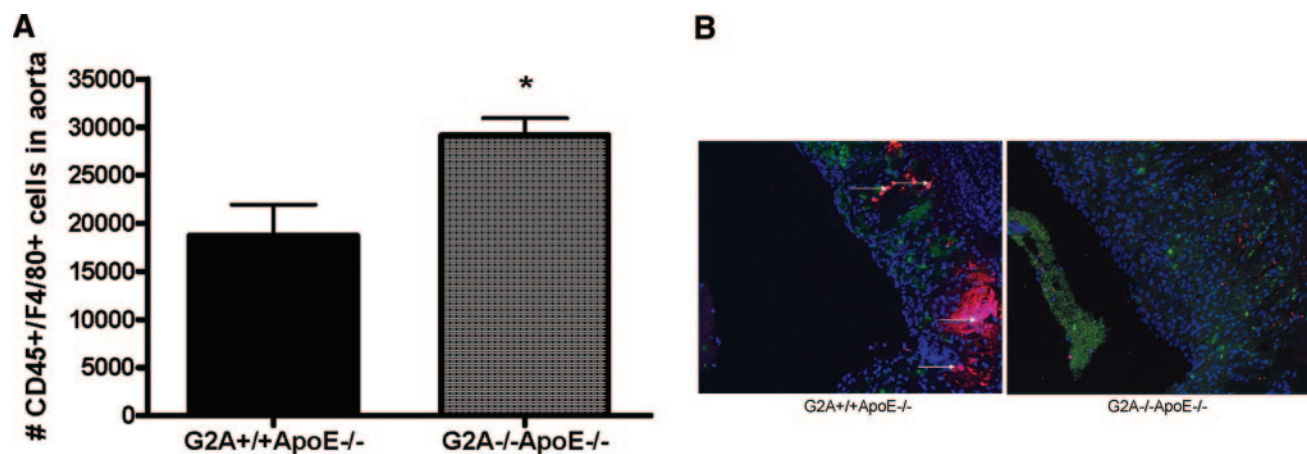


Figure 8. Increased numbers of macrophages in the aortic wall of G2A^{-/-}ApoE^{-/-} mice. **A**, Whole aorta flow cytometry for macrophage content. Aortas were isolated from 10 each of G2A^{+/+}ApoE^{-/-} and G2A^{-/-}ApoE^{-/-} mice after 10 weeks on Western diet. Aortas were digested and analyzed for leukocyte content by flow cytometry as described in Materials and Methods. Data were analyzed using FlowJo software and the total number of CD45⁺F4/80⁺ cells/aorta was plotted. * $P < 0.005$, significantly greater than G2A^{+/+}ApoE^{-/-} control. **B**, Fluorescent imaging of lesional macrophage apoptosis. Five-micron aortic root sections at +160 μm from the transition were stained for resident macrophages and apoptosis. Green indicates MAC-2; red, TUNEL; blue, DAPI nuclear stain. G2A^{-/-}ApoE^{-/-} aortic root sections showed increased individual macrophage staining and decreased TUNEL. G2A^{+/+}ApoE^{-/-} sections showed increased MAC-2/TUNEL colocalization to nuclear intact cells as indicated by the arrows (pink color).

In related studies of G2A function, Parks et al examined G2A-deficient mice on a LDLR^{-/-} background and noted increased macrophage content and decreased macrophage apoptosis, with no effect on atherosclerotic lesion size after either 6 or 12 weeks of Western diet feeding.²¹ A second study from the same group demonstrated that G2A deficiency decreased atherosclerosis in G2A^{-/-}Ldlr^{-/-} mice at later time points of diet feeding.³⁵ In the second study, these investigators reported significant elevations in plasma HDL levels in the G2A^{-/-}Ldlr^{-/-} mice when fed a Western diet for both 9 and 20 weeks. They observed a significant decrease in atherosclerosis in G2A^{-/-}Ldlr^{-/-} mice at both time points of feeding that could possibly be attributed to increased plasma HDL concentrations. Our studies were performed in ApoE^{-/-} mice for 10 weeks, and we did not observe changes in HDL. Moreover, we performed bone marrow transplantation studies in both genetic backgrounds and found that deficiency of G2A in bone marrow-derived cells significantly contributed to atherosclerosis (Figure 7). In the apoE^{-/-} background, this trend did not reach statistical significance, most likely because of the small number of animals available for study. However, the effect was quite dramatic in the Ldlr^{-/-} mice, and our findings are opposite to those of Parks and colleagues.^{21,35} HDL levels were similar among groups in our bone marrow transplant studies (data not shown), which is different from the studies by Parks and colleagues. However, taken together, the collective results of our studies and those of Parks et al suggest that G2A expression in multiple cell types influences atherosclerosis. Indeed, absence of G2A in hepatocytes, lymphocytes, and endothelium clearly influences inflammatory and immune processes related to atherosclerosis.^{22,41,34} Development of floxed mice for cell-specific studies of G2A deficiency is needed to dissect the important contributions of G2A in each cell type on atherosclerosis.

G2A expression has been shown to influence apoptosis in leukocytes.^{16,21,36} Recent studies suggest an important role of apoptosis in atherosclerotic plaque formation.^{37,38} Our previous study demonstrated that G2A deficiency resulted in increased NFκB activation in murine aortic endothelial cells.²² Because NFκB activation targets survival gene expression resulting in decreased apoptosis,³⁹ we investigated whether this was relevant in macrophages in the present study. Indeed, G2A^{-/-}ApoE^{-/-} macrophages showed significantly higher levels of NFκB p65 expression in the nucleus that corresponded with increased survival gene expression. Expression of NFκB-controlled genes that are important for cell survival, including bcl-2, bcl-xL, TRAF, and cFLIP was elevated in macrophages from G2A^{-/-} mice. These G2A-deficient macrophages also showed significantly lower TUNEL, cleaved caspase-3, and Annexin V staining. Furthermore, we observed downregulation of the proapoptotic genes, p53 and p73, which have been shown to be downregulated by NFκB.⁴⁰ Concomitantly with the increase in prosurvival genes, we observed increased numbers of macrophages in the aortic wall of G2A^{-/-} mice in vivo, suggesting that G2A

deficiency causes macrophage accumulation in the aortic wall through promoting macrophage survival. We speculate from our data that NFκB activation is the primary regulator of macrophage survival in the absence of G2A. Because we have observed activation of NFκB in endothelium as well, we anticipate that G2A expression somehow serves to inhibit NFκB. The mechanisms for this are unknown but are presently being studied in the laboratory. In preliminary studies, we have not observed significant changes in IκB expression (data not shown), but we have observed increased AKT activity in the G2A^{-/-}ApoE^{-/-} macrophages, which has been shown to increase NFκB.⁴¹ However, we cannot rule out contributions of other survival pathway genes in regulating apoptosis in the G2A^{-/-} macrophages. Moreover, we found upregulation of a few proapoptotic genes, including FasL, and caspase-12 in G2A^{-/-} macrophages, which suggest that the macrophages in G2A^{-/-} mice have become dysregulated, most likely impacting their inflammatory phenotype as well. Indeed, caspase-12 is induced by endoplasmic reticulum stress, which often occurs as a result of free cholesterol loading in macrophages.⁴² Endoplasmic reticulum stress can contribute to a proinflammatory macrophage phenotype.³⁸

Under normal conditions, macrophages act to maintain homeostasis in the aortic wall. Classically activated (M1-type) macrophages exhibit strong microbicidal properties, thereby promoting IL-12 and TNFα-mediated Th1 responses.⁴³ In contrast, alternatively activated (M2-type) macrophages secrete antiinflammatory cytokines such as transforming growth factor β, ingest and clear cell debris, and are rapidly cleared from the wall, thereby contributing to the resolution of inflammation. In normal tissue and during early atherosclerosis, M2-type macrophages help to stabilize the environment of the vessel wall by promoting effective efferocytosis of dying cells.⁴⁴ Chronic activation of M1-like macrophages promotes an unstable vessel environment, reducing efferocytosis and triggering secondary necrosis of vascular wall cells, thereby contributing to the advanced atherosclerotic plaque formation.³⁸ G2A has recently been identified as a phagocyte receptor on macrophages, in which G2A recognizes “find me” signals such as lysophosphatidylcholine metabolites that are secreted by dying cells. The increased collagen content observed in aortic roots of G2A-deficient mice (Figure 6) suggests that there is increased secondary necrosis of vascular wall cells that contribute to plaque complexity. This finding is consistent with the notion of Peter et al that G2A may serve as a phagocytic receptor.⁴⁵ Thus, the absence of G2A on macrophages may contribute to atherosclerosis through impairing efferocytosis pathways, in which various find me signals secreted by dying cells are no longer recognized by the macrophage in the absence of G2A. We did find reductions in apoptotic cell engulfment in G2A^{-/-} macrophages in vitro, supporting this hypothesis (Figure 3). Moreover, defective apoptotic cell clearance has been linked to atherosclerosis in mice.²⁶ Future studies to determine the specific roles of the G2A receptor in apoptotic cell clearance and its subsequent impact on atherosclerosis will be needed to fully address this hypothesis.

In conclusion, we demonstrate that G2A deficiency caused increased atherosclerosis in the aortic sinus of ApoE^{-/-} mice fed a Western diet. We propose this is attributable to increased numbers of lesional macrophages and decreased macrophage apoptosis in the aortic wall. These findings demonstrate that G2A deficiency triggers an abnormal inflammatory macrophage phenotype that contributes to atherosclerosis development, suggesting a critical role of G2A in macrophage homeostasis.

Note Added in Proof

After submission of this manuscript, Frasch et al reported that antibody blockade of the G2A receptor reduces apoptotic cell uptake by macrophages, further confirming our engulfment data in G2A-deficient macrophages.

Frasch SC, Berry KZ, Fernandez-Boyanapalli R, Jin HS, Leslie C, Henson PM, Murphy RC, Bratton DL. NADPH oxidase-dependent generation of lysophosphatidylserine enhances clearance of activated and dying neutrophils via G2A. *J Biol Chem*. 2008;28283:33736–33749.

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Disclosures

None.

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Supplement Material

Materials and Methods

Reagents. Cytometric bead arrays (#552364) were from BD Biosciences.

Antibodies used were: NF κ Bp65, β -actin, histone, MMP-9 (Santa Cruz #s SC-372, SC-47778, SC-8030, SC-6840, respectively), GSK3 β , phospho-NF κ Bp65 (Cell Signaling #s 9661, 9242, 9332, 3031, respectively), MAC-2 (Cedarlane), streptavidin and anti-rabbit Alexa secondary antibodies (Molecular Probes).

TMR TUNEL *in situ* cell death reagent was from Roche Applied Sciences.

Mice: ApoE-deficient (ApoE $^{-/-}$) mice (stock #002052) and LDL receptor-deficient (Ldlr $^{-/-}$) mice (stock # 002207) were purchased from the Jackson Laboratory.

G2A-deficient (G2A $^{-/-}$) mice were generated by Witte and colleagues¹ and backcrossed for 14 generations onto the C57BL/6 background. G2A $^{-/-}$ mice were subsequently backcrossed onto the ApoE $^{-/-}$ or Ldlr $^{-/-}$ backgrounds.

G2A $^{+/+}$ ApoE $^{-/-}$ and G2A $^{-/-}$ ApoE $^{-/-}$ female mice were maintained on normal chow diet or fed a Western-type diet (Teklad #88137) for ten weeks. All animal studies were performed following approved guidelines of the University of Virginia Animal Care and Use Committee.

Bone marrow transplantation studies. Ldlr $^{-/-}$ and apoE $^{-/-}$ recipient mice were irradiated in two doses of 550–600 rads each, for a total of 1100–1200 rads, \approx 4 h apart. Bone marrow cells from both femurs and tibias of donor mice (apoE $^{-/-}$, G2A $^{-/-}$ apoE $^{-/-}$, Ldlr $^{-/-}$, and G2A $^{-/-}$ Ldlr $^{-/-}$) were harvested under sterile conditions.

Approximately 50 million nucleated bone marrow cells were obtained from each donor mouse. Bones were flushed with RPMI (Life Technologies, Grand Island, NY) (without phenol red) with 10% FCS (Atlanta Biologicals, Norcross, GA). Suspended bone marrow cells were washed and lysed in 1.5 mM NH₄Cl lysing solution. Approximately 1–2 million unfractionated bone marrow cells in 200 µl of media were delivered i.v. through the tail vein of each recipient mouse. Recipient mice were housed in a barrier facility under pathogen-free conditions before and after bone marrow transplantation. After bone marrow transplantation, the mice were maintained on autoclaved water with antibiotics (0.7 mM neomycin sulfate, 60 µM tetracycline, and 0.37 mM trimethoprim) (Sigma, St. Louis, MO) and fed autoclaved food. Mice were used for experiments after 6 weeks of bone marrow reconstitution.

Atherosclerosis Quantification. Atherosclerosis was quantified in aortic roots and throughout the aortic arch using the en face technique as described previously^{2,3}.

Isolation of peritoneal macrophages. Peritoneal macrophages were isolated by peritoneal lavage 5 days post-injection of 2mL 4% thioglycolate media as previously described⁴.

Quantitative real-time PCR. Macrophages were freshly isolated by peritoneal lavage 5 days post thioglycolate injection. Total cellular RNA was obtained from macrophages as described previously⁵, and cDNA was synthesized from 1mg RNA using iScript (Biorad). For quantitative PCR analyses of survival gene mRNA abundance, cDNA was diluted 1:8; 4 µL of this dilution were used for each

PCR reaction. Reagents from the BioRad real-time PCR kit containing Sybr Green were used for quantitative PCR reactions. cDNA (2 μ l) from each experimental group were used. Primer sequences used were as follows: IAP: sense 5'-AAT GAAGAACTAAGGATGGACACG -3' antisense 5'-ACTTGTTGTGCTCTTGTATTGGG-3', cFLIP: sense 5'-ATC TGGTGATTGAATTGGAG-3' antisense 5'-ATATGATAGCCCAGGGAA GT-3', BCL-2: sense 5'-CCTTCCAGCCTGAGAGCAAC-3' antisense 5'-ACG ACGGTAGCGACGAGAG -3', BCL-xL: sense 5'-AGCGTTCAGTGATCT AATCC-3' antisense 5'-GCAATCCGACTCACCAATACC-3', TRAF: sense 5'-ACCCACCTGTCCCTCTTCATC G-3' antisense 5'-TCTCGGTTGTTCTGGTCAAGTAGC-3', and IL-6 5'-CTGCAAGAGACTTCCATCCAGTT-3' antisense 5'-AGGGAAGGCCGTGGTTGT-3'. TNF α , iNOS, IL-12p40, Arginase-1, TGF β , and KC primer sequences used were as previously reported ⁴. PCR for murine β -actin was performed as a control: sense F: 5'-AGCACTGTGTTGGCGTACAG-3' and R: 5'-CTCTTCCAGCCTTCCTTCCT-3'. The PCR conditions were: 95 °C 10 min, 95 °C 4 min, followed by 40 cycles of 95 °C 15 secs, 55 °C 30 secs, 72 °C 30 secs, followed by a final extension at 72°C for 15 secs. Data were analyzed and presented based upon the relative expression method ⁶. This formula for calculation is:

$$\text{Relative expression} = 2^{-(\Delta\Delta CT)}$$

where ΔC_T is the difference in threshold cycle between the gene of interest (i.e., cFLIP) and the housekeeping gene (β -actin). In this equation, **S**= G2A^{-/-}ApoE^{-/-} mouse and **C**= G2A^{+/+}ApoE^{-/-} mice.

Dad1, IAP2, FasL, PAK7, Caspase12, p73 and p53 were measured using the RT²Profiler PCR array for mouse apoptosis (Super Array Corp.) according to manufacturer's instructions and were verified in additional samples using real-time PCR.

Flow cytometry. Peritoneal macrophages were isolated from mice as described above. Macrophages were pelleted by centrifugation at 200xg for 5 minutes and resuspended in FACs buffer containing 5% fatty acid free bovine serum albumin in PBS with Fc blocker (Calbiochem). Cells were stained with antibody for 20 minutes at 4°C in the dark. Cells stained for annexin V were prepared according to manufacturer's instructions (Molecular Probes). After gating on the macrophage population, 150,000 cells per sample were analyzed for each antibody as described previously⁷. Samples were analyzed at the University of Virginia Flow Cytometry Core using a Becton Dickinson FACSCaliburTM instrument.

Whole aorta flow cytometry. FACs analysis was performed on whole aorta as described previously^{8;9}. Briefly, perfused aortas were excised from the heart to the abdominal bifurcation, cleaned, and digested in 125 U/ml collagenase type XI, 60 U/ml hyaluronidase type I-s, 60 U/ml DNase1, and 450 U/ml collagenase type I in PBS containing 20 mM Hepes at 37°C for 1 h. A single cell suspension was obtained by gently forcing the aorta digest through a 70- μ M strainer. Cells

were incubated with antibodies for 20 min at 4°C, washed twice, subsequently stained for annexin V for apoptosis studies and analyzed by flow cytometry on a Becton Dickinson FACSCalibur™ instrument. Data analysis was performed using FlowJo software.

Apoptotic Cell Engulfment Assays. Engulfment assays were performed as described ¹⁰. Jurkat T cells were labeled with Cell Tracker Red (Invitrogen) and exposed to UV light for 15 minutes to induce apoptosis. After incubation at 37°C for 1h, 50% of these cells are apoptotic (Annexin V⁺, PI⁻). Apoptotic Jurkat cells were washed with PBS and added to macrophages for 15 minutes. At the end of incubation period, cells were washed several times with ice cold PBS.

Macrophages were removed by trypsin-EDTA solution and the fraction of mφs containing apoptotic cell-derived red fluorescence was measured using flow cytometry.

Immunoblotting. Cells were harvested and lysed in a modified RIPA lysis buffer containing 50mM TrisHCl (pH8.0), 150mM NaCl, 1% NP40, 10mM NaF, 2mM Na₃VO₄, and protease inhibitors. After centrifugation, protein extract supernatant was collected. 50 μg of MAEC membrane lysate protein was analyzed by 4-12% SDS-PAGE in MOPS running buffer and transferred to nitrocellulose. Pierce Blocker BLOTTO in TBS was used as a blocking agent. Membranes were probed with a 1:1000 dilution of G2A mouse monoclonal antibody and a 1:4000 dilution of anti-mouse IgG-HRP (Amersham). Blots were stripped and reprobed with 1:4000 tubulin and 1:10,000 anti-mouse IgG-HRP secondary and quantitated using densitometry.

For NF κ B studies, peritoneal macrophage lysate from G2A^{+/+}ApoE^{-/-} and G2A^{-/-}ApoE^{-/-} mice was harvested and nuclei and cytosolic extracts were collected using the NE-PER kit (Pierce) according to manufacturer's instructions. Immunoblotting was performed as described previously¹¹. Anti-NF κ B p65 antibody was used at 1:2000 dilution. HRP-conjugated anti-rabbit secondary antibody (Amersham) was used at 1:4000 dilution.

Inflammatory cytokines. Plasma from 6 each of G2A^{+/+}ApoE^{-/-} and G2A^{-/-}ApoE^{-/-} mice was obtained as described above. Plasma concentrations were determined for interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1), interferon γ (IFN- γ), TNF α , and interleukin-12p70 (IL-12p70) using a Cytometric Bead Array according to manufacturer's instructions (BDBiosciences).

Histology. Immunofluorescence. For measurement of apoptosis *in vitro*, peritoneal macrophages were plated in chamber slides. After 24 hours in culture, cells were fixed, permeabilized, and stained for TUNEL or Caspase-3 and nuclei using DAPI as described previously¹². For measurement of apoptosis and atherosclerosis *in vivo*, aortic roots were isolated from mice and fixed in 4% paraformaldehyde in PBS overnight. Tissues were processed and embedded in paraffin at the University of Virginia Histology Core. To measure macrophage content and apoptosis levels in aortic root, sections (5 μ M) were immunostained for MAC-2 (Cedarlane) or TUNEL (Roche Applied Sciences), respectively, and mounted using Vectashield hard mount with DAPI for nuclei staining (Vector

Laboratories). Secondary fluorescent antibody for MAC-2 was from Molecular Probes.

Immunohistochemistry. Sections (5 μ M) were stained using Movats pentachrome (Color key: nuclei - blue to black, cytoplasm – red, elastic fibers - dark purple to black, collagen and reticulum fibers - yellow to greenish yellow, ground substances and some reticulum fibers - blue to bluish-green, fibrinoid - intense red)¹³, or picosirius red. Under normal light, collagen content of the lesion is visible by red staining. Under polarized light, red, orange, yellow, and green colors are apparent (the colors of collagen fibers in order of decreasing thickness)¹⁴.

Statistical Analyses: Data for all experiments were analyzed using the StatView 6.0 software program. Comparisons between groups were performed using one-way analysis of variance (ANOVA) methods. Data are graphically represented as mean \pm SE, in which each mean consists of 3 or 4 experiments performed in triplicate using a minimum of ten mice per group (unless noted otherwise in the figure legends). Comparisons between groups and tests of interactions were made assuming a two-factor analysis with the interaction term testing each main effect with the residual error testing the interaction.

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