Published in final edited form as:

Atherosclerosis. 2020 November ; 313: 26–34. doi:10.1016/j.atherosclerosis.2020.09.018.

Maternal exposure to soy diet reduces atheroma in hyperlipidemic F1 offspring mice by promoting macrophage and T cell anti-inflammatory responses

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Abstract

Background and aims.—Maternal hypercholesterolemia has been implicated in the earlier onset of atherosclerotic lesions in neonatal offspring. In this study, we investigated whether maternal exposure to soy protein isolate (SPI) diet attenuated the progression of atherosclerosis in F1 offspring.

Method: Pregnant apolipoprotein E knockout (*Apoe*^{−/−}) female mice were fed SPI diet until postnatal day 21 (PND21) of the offspring (SPI-offspring). SPI-offspring were switched at PND21 to casein (CAS) diet until PND140. Mice, fed CAS throughout their lifetime (gestation to adulthood), were used as controls (CAS-offspring).

Results: Atherosclerotic lesions in the aortic sinuses were reduced in SPI-offspring compared with CAS-offspring. Total serum cholesterol levels in CAS-offspring or dams were comparable to levels in their SPI- counterparts, suggesting that alternative mechanisms contributed to the athero-protective effect of maternal SPI diet. Aortic VCAM-1, MCP-1, and TNF- α mRNA and protein expression, and expression of macrophage pro-inflammatory cytokines were reduced in SPI-offspring. Interestingly, CD4⁺ T cells from SPI-offspring showed reduced IFN- γ expression

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Author contributions: SV and SN conceived and designed the experiments. RLB, SV, BP performed experiments. RLB, SV, BP, and SN analyzed the data. PF and SN wrote the manuscript with contribution from other authors.

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Conflict of interest: The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

(Th1), while the expression of IL-10 (Th2/Treg), and IL-13 (Th2) was increased. DNA methylation analyses revealed that anti-inflammatory T cell-associated genes such as *Gata3* and *Il13* promoter regions were hypomethylated in SPI-offspring. These findings suggest that anti-inflammatory macrophage and T cell response could have contributed to the athero-protective effect in SPI-offspring.

Conclusions: Our findings demonstrate that gestational and lactational soy diet exposure inhibits susceptibility to atherosclerotic lesion formation by promoting anti-inflammatory responses by macrophages and T cells.

Graphical Abstract

Keywords

Gestation; Inflammation; maternal hypercholesterolemia; soy protein; atherosclerosis

INTRODUCTION

The “fetal origins of adult disease” hypothesis refers to the theory that *in utero* environment may be a vital contributor to the predisposition for chronic diseases in offspring¹. The occurrence of fatty streaks in the aorta, and the progression of subclinical pediatric coronary atherosclerosis to cardiovascular disease (CVD) in adulthood, are becoming increasingly clear^{2, 3}. Maternal hypercholesterolemia has been implicated in a higher incidence of

atherosclerotic lesions during the fetal period, and faster progression of these atherosclerotic lesions after birth when compared with controls^{2–5}. These clinical observations are supported by animal studies showing maternal hypercholesterolemia-induced atherosclerotic lesions in F1 offspring^{6–9}. Notably, maternal consumption of trans-fatty acids promoted atherosclerosis in the F1 offspring¹⁰. These key findings suggest that maternal diet, including hypercholesterolemia, initiates the atherogenic process during fetal development.

A series of inflammatory processes involving the vascular endothelium and migration of monocytes into the vascular intima are integral to the progression of atherosclerosis^{11, 12}. Pro-inflammatory cytokines like TNF- α induce the upregulation of cellular adhesion molecules, such as E- and P-Selectins, VCAM-1, and ICAM-1^{13, 14}. Concurrently, activation of monocytes by pro-inflammatory cytokines or oxidized lipids induces firm adhesion to the activated endothelial cells. Chemokines such as monocyte chemoattractant protein (MCP-1, gene *Ccl2*) then promote the transmigration of monocytes to the vascular intima¹⁵. Once monocytes are within the vascular intima, they are transformed into macrophages, which subsequently take up oxidized-low density lipoprotein (oxLDL)¹⁶ to initiate the formation of lipid-laden macrophages (also known as foam cells) within the intima. In addition to macrophages, T cell migration from lymphoid organs also contributes to the progression of atherosclerosis^{12, 17–19}.

Epidemiological studies have shown a lower incidence of CVD in Asia than in Western countries²⁰. Studies by us²¹ and others^{22, 23} have demonstrated that spontaneously hyperlipidemic apolipoprotein E knockout (*Apoe*^{−/−}) mice weaned on soy protein isolate (SPI) diet or dietary β -conglycinin, one of the major soy proteins, showed reduced atherosclerotic lesions. Based on the athero-protective effect of postnatal dietary SPI exposure, we aimed to determine whether exposing developing F1 mouse pups to SPI diet during early development (gestational and lactational periods) could attenuate the development of atherosclerotic lesions in adulthood.

We hypothesized that F1 offspring with a genetic predisposition for atherosclerosis, when exposed in utero to SPI diet, would show decreased susceptibility to atherosclerosis. This hypothesis was investigated using hyperlipidemic *Apoe*^{−/−} female mice fed SPI diet during gestation/lactation and evaluated the progression of atherosclerosis in the F1 offspring. We also investigated the underlying mechanism(s) of the athero-protective effects of the SPI diet during early development. Using the hyperlipidemic mouse model, we showed that maternal exposure to the SPI diet attenuated atherosclerotic lesions in F1 offspring. Interestingly, maternal exposure to SPI diet-induced anti-inflammatory macrophage and T cell responses in the offspring.

MATERIALS AND METHODS

Animal studies.

Female (N=8) and male (N=8) *Apoe*^{−/−} mice (Jackson Laboratory, Bar Harbor, ME) were housed in micro-isolator cages and maintained on a 12h light/dark cycle in a temperature-controlled room. Male and female *Apoe*^{−/−} mice (6 wk.) were paired at a 1:1 ratio and were fed either CAS (N=4, female) or SPI (N=4, female) diets (Supplemental Table 1). The semi-

purified diets were made according to the AIN-93G diet formula²¹, with the exception that corn oil replaced soybean oil, and the protein source was either CAS (New Zealand Milk Products, Santa Rosa, CA) or SPI (The Solae Company, St. Louis, MO). Pups were exposed to the maternal diet (CAS or SPI) until weaning (Fig. 1A). At weaning (postnatal day 21, PND21), pups (both male and female) from CAS-fed dams (hereafter referred to as CAS-offspring) were separated into holding cages and fed CAS diet. Pups from SPI-fed dams (SPI-offspring) were switched to the CAS diet until PND42 or PND140 (Fig. 1A). Mice consumed food and water ad libitum throughout the study period. Litter size and growth rates of F1 offspring were monitored to determine the effect of maternal exposure to SPI on postnatal development. Animals were euthanized with isoflurane by inhalation, followed by CO₂ asphyxiation. The hearts and descending aortas were used to analyze aortic sinus lesions and gene expression, respectively. Liver, spleens, and resident peritoneal macrophages were collected for gene expression and CD4⁺ T cell responses. These studies were conducted under the guidelines for the care and use of laboratory animals by the United States, and the Institutional Animal Care and Use Committees at the University of Arkansas for Medical Sciences, and the University of Pittsburgh approved all the animal use protocols.

Atherosclerotic lesion analysis.

Aortic sinus cryosections (8 μ m) were stained with Oil Red O according to methods described previously^{24, 25}. The mean lesion area in each of five serial sections from each mouse was obtained, and quantitative analysis of atherosclerosis was performed as described^{24, 25}.

Immunohistochemistry.

Serial aortic sinus cryosections (8 μ m) were stained with biotin-conjugated rat anti-mouse macrophage antibody (MOMA-2, Rat IgG2b, 1:25 dilution, AbD Serotec, Raleigh, NC), followed by either goat anti-mouse VCAM-1 (1 μ g/ml, RND systems, Minneapolis, MN), or rat anti-MCP-1 (clone 123616, rat IgG2b, RND systems) antibodies. Images of VCAM-1 and MCP-1 immunopositive areas were acquired using Axiovision software.

Analysis of serum lipids.

Blood samples were collected from F1 offspring in heparin-coated tubes at PND140, using cardiac puncture. Maternal blood samples were acquired before pregnancy (one day before mating) and at the time of weaning the pups at PND21, to determine maternal plasma total cholesterol levels. All mice were fasted 16 h before blood collection. Concentrations of plasma total cholesterol, HDL-cholesterol, and triglycerides were quantified using enzymatic kits from Synermed (Montreal, Canada) as previously described^{21, 24}.

T cell subsets.

Spleens were excised from CAS- or SPI-offspring (at PND140) to determine T cell (Th1, Th2, Th17, and Treg) cytokine response. CD4⁺ T lymphocytes were purified from splenocytes by negative selection using anti-CD4 microbeads (Miltenyi, Auburn, CA). Purified CD4⁺ T cells (1 $\times 10^6$ /well) were activated with plate-bound anti-CD3 mAb (5

µg/mL, clone 145–2C11, BD Biosciences, San Diego, CA) in the presence of soluble anti-CD28 mAb (1 µg/mL, clone 37.51, BD Biosciences) for 48 h²⁵. Supernatants collected after T cell stimulation were stored at –70°C for cytokine analysis. Cells were then lysed, and RNA was prepared for Th1/Th2 gene expression analyses by quantitative RT-PCR (qRT-PCR).

Cytokine analysis.

Cytokines secreted by activated T cells were analyzed using a mouse Th1/Th2/Th17 (IL-2, IL-4, IL-6, IFN-γ, TNF-α, IL-10, and IL-17A) BD-cytokine bead array (BD-Biosciences) according to the manufacturer's instructions. Beads were analyzed using the FACSCalibur flow cytometer and FCAP array (BD Biosciences).

Gene expression.

Aortas were perfused with nuclease-free PBS, and total RNA was isolated from a proximal portion of the descending aorta, including the aortic arch with brachiocephalic arteries, using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. *Vcam1*, *Mcp1*, and *Tnfa* expression in the aorta, and expression of antioxidant enzymes in the liver were determined by quantitative RT-PCR (qRT-PCR) after reverse transcription of total RNA (0.5 µg), followed by PCR using specific primer pairs (SA-Biosciences, Frederick, MD), as described earlier²⁴. Expression of Th1/Th2-related genes in the aortas and CD4+ T cells was determined using a Th1/Th2 RT-PCR array (SA-Biosciences), as previously described²⁵. The expression of target genes was calculated using the Ct method, using threshold cycles for β-actin and GAPDH as normalization references. All qRT-PCR reactions were carried out at least twice from independent cDNA preparations. cDNA preparations using RNA without reverse transcriptase served as negative controls.

Macrophage inflammatory response.

Peritoneal lavages were collected (at PND42 and PND140) by washing the peritoneum with 10 ml of ice-cold PBS with 1 mM EDTA and filtered through 100-µm nylon cell strainers to remove debris. Cells were resuspended in RPMI 1640 medium containing 2% FBS and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin), and then plated in 12-well culture plates (2 × 10⁶ cells/well) and incubated for 2 h at 37°C in a 5% CO₂ incubator. Non-adherent cells were removed by washing the plates three times with RPMI 1640 medium, and the adherent resident peritoneal macrophages (PND42) were cultured overnight before preparing lysates to isolate RNA. RNA was prepared using the RNeasy kit (Qiagen), and mRNA expression levels of pro-inflammatory genes were determined by real-time qRT-PCR using specific primer pairs (SA-Biosciences), as previously described²⁴.

DNA methylation.

Genomic DNA was isolated from CD4+ T cells (PND42) using the QIAamp DNA Mini Kit (Qiagen), and DNA was quantified using the Nanodrop spectrophotometer. Methylation of the promoter regions of 24 genes involved in inflammatory response was quantified using the EpiTect Methyl qPCR Array Inflammatory Response and Autoimmunity Array (SA-Biosciences), according to manufacturer recommendations. Genomic DNA (1 µg) from each

sample was treated with the methylation-sensitive restriction enzyme A or methylation-dependent restriction enzyme B (EpiTect methyl II DNA restriction kit). Following digestion, the remaining DNA in individual enzyme reaction was quantified by real-time PCR, using primers that flank one of the promoter regions of each of the genes tested. The relative fractions of methylated and unmethylated DNA were subsequently quantified by comparing the amount in each digest with that of a mock (no enzymes added) digest, using the Ct method. Data were analyzed using an integrated Excel-based template (SA-Biosciences), which normalized the Ct values of both digests with the mock-digest values to calculate the percentages of methylated and unmethylated CpG DNA.

Statistical analysis.

Differences between diet groups for the indicated parameters were analyzed by unpaired two-tailed Student's *t*-tests using GraphPad Prism 8.4 for Macintosh (GraphPad Software, San Diego, CA). Results were expressed as means $\pm$ SD. Differences were considered significant at $P < 0.05$.

RESULTS

Maternal soy exposure does not affect litter size or growth.

Weight gain during gestation was not different between pregnant female *Apoe*^{-/-} mice fed CAS diet and those fed SPI diet (data not shown). Maternal dietary SPI exposure did not affect litter size, male to female ratio, or birth weight of SPI-offspring (Supplemental Table 2). Bodyweight gain was not different between male and female F1 offspring of SPI- and CAS-fed dams (Fig. 1B). Bodyweight gains between 30 and 60 days in male and female SPI-offspring were lower compared to CAS-offspring. However, the difference was not significant ($p=0.45$). At PND140, liver, kidney, and spleen weights were not different between CAS- and SPI-offspring (data not shown). These findings suggest that maternal SPI exposure did not affect the growth and development of F1 offspring.

Apoe-deficient F1 mice exposed to SPI during early development show attenuated lesions.

We determined whether maternal SPI exposure inhibits the progression of atherosclerosis. Analyses of the aortic sinus showed that maternal SPI exposure reduced atherosclerotic lesions in SPI female offspring (Fig. 1C). Quantification of atherosclerotic lesions in the aortic sinus of SPI female offspring mice showed 50% reduced lesions compared with female CAS-offspring (Fig. 1D). Similar findings were observed in male SPI-offspring compared to male CAS-offspring (Fig. 1E and F), suggesting that there is no gender bias in the athero-protective effect of maternal soy protein exposure.

Maternal SPI feeding does not change plasma lipid levels.

Plasma total- and HDL-cholesterol, and triglyceride levels in female SPI-offspring (at PND140) were not different from those in female CAS-offspring (Supplemental Fig 1A–C). Though triglyceride levels in SPI-offspring were lower than in CAS-offspring, the difference was not statistically significant ($p=0.18$). Similar findings were observed in male CAS- and SPI-offspring (data not shown). Moreover, maternal cholesterol levels of pregnant *Apoe*^{-/-} mice fed CAS or SPI diet during mating (Supplemental Fig 1D), and at the time of weaning,

the pups (Supplemental Fig 1E) were similar. These findings suggest that maternal exposure to the SPI diet that reduces atherosclerotic lesions in SPI-offspring is dependent on mechanisms other than regulating cholesterol homeostasis.

Mechanisms contributing to the athero-protective effect of maternal SPI diet.

Oxidative stress has been suggested to contribute to the progression of atherosclerosis^{26,27}. Hence, we investigated whether antioxidant pathways mediate reduced atherosclerosis in SPI-offspring. Real-time quantitative RT-PCR analyses of the liver from SPI offspring showed that mRNA expression of antioxidant enzymes was not different between SPI- and CAS-offspring (Supplemental Fig 2). These findings suggest that reduced atherosclerosis in SPI-offspring is independent of regulating the antioxidant pathway.

Then, we investigated whether the potential anti-inflammatory mechanisms mediate the attenuation of lesions in SPI-offspring female mice have been shown to develop more atherosclerotic lesions than age-matched males^{28, 29}, we used tissue samples collected from female F1 offspring to assess possible molecular mechanisms contributing to athero-protective effects of gestational/lactational exposure to SPI. Real-time qRT-PCR analyses showed that *Vcam1* expression in the lesion-prone region of the proximal aorta (aortic arch to the thoracic aorta) was decreased by 60% ($P < 0.001$) in SPI-offspring, compared to CAS-offspring (Fig. 2A). Aortic sinuses of SPI-offspring also showed reduced VCAM-1 immunopositive areas (Fig. 2B and C).

We then investigated the expression of TNF- α and MCP-1, a pro-inflammatory cytokine, and chemokine, which have been implicated in the initiation and progression of atherosclerosis^{30, 31}. MCP-1 mRNA expression was 50% lower ($P < 0.01$) in aortas from SPI-offspring (Fig. 3A). Similarly, mRNA levels of the pro-inflammatory cytokine TNF- α were reduced by 50% ($P < 0.001$) in aortas from SPI-offspring (Fig. 3B). Reduced MCP-1 mRNA expression also paralleled the attenuated aortic MCP-1 immunopositive area in SPI-offspring (Fig. 3C and D). We then determined whether attenuated VCAM-1 and MCP-1 expression resulted in reduced migration of monocytes and subsequent transformation of macrophages. Immunohistochemical analyses of the aortic sinus showed that in SPI-offspring had fewer macrophages compared to CAS-offspring (Fig. 3E).

Reduced pro-inflammatory cytokine expression in macrophages from SPI-offspring.

Pro-inflammatory cytokine and chemokine responses initiated by macrophages lead to the progression of atherosclerosis¹⁵. We, therefore, assessed the effect of maternal SPI exposure on macrophage pro-inflammatory gene expression at PND140. Resident peritoneal macrophages from CAS-offspring showed elevated expression of *Mcp1*, *Tnfa*, and *Il6* (Supplemental Fig. 3A–C). Interestingly, macrophages from SPI-offspring showed about 60–80% lower expression of these pro-inflammatory genes, compared to macrophages from CAS-offspring (Supplemental Fig. 3A–C). Macrophages from SPI-offspring at PND42 also showed reduced expression of pro-inflammatory cytokine and chemokines at PND42 (Supplemental Fig. 3D–I). These studies suggest that gestational and lactational soy exposure reduced pro-inflammatory cytokine expression in macrophages.

Maternal SPI exposure promotes athero-protective T cell response in SPI-offspring.

Recent studies have suggested that an imbalance between pro-inflammatory (Th1 and Th17) and anti-inflammatory (Th2 and Treg) T cell responses contribute to the progression of atherosclerosis^{18, 19}. Activated CD4⁺ T cells showed downregulated *Ifng*, *Il12*, and *Tnfa* mRNA expression (Th1 cytokines) in SPI-offspring compared to CAS-offspring (Fig. 4A–C). Notably, *Il4*, *Il13* (Th2 cytokines), and *Il10* (Treg) mRNA expression levels were about 2- to 5-fold higher in SPI-offspring (Fig. 4D–F). To address whether changes in splenic T cell cytokine mRNA expression were also reflected in the lesion-prone aortic tissue, we analyzed the mRNA expression levels of *Ifng* and *Il10* in the aortas of CAS- and SPI-offspring. *Ifng* mRNA expression in the aorta (at the lesion site) was lower in SPI-offspring than in CAS-offspring (Fig. 4G). Consistent with the changes in cytokine mRNA levels in splenic CD4⁺ T lymphocytes, mRNA levels of *Il10* in the aortas of SPI-offspring were significantly higher (3-fold) than in CAS-offspring (Fig. 4H).

SPI-offspring show increased anti-inflammatory T cell response.

We then investigated cytokine secretion by CD4⁺ T in the SPI offspring. Activated CD4⁺ T cells from CAS-offspring secreted high levels of pro-inflammatory Th1 cytokines IFN- γ , TNF- α , and IL-6, and Th17 cytokine IL-17 (Fig. 5A–D) compared to cytokine secretion by CD4⁺ T cells from SPI-offspring. Conversely, activated CD4⁺ T cells from SPI-offspring secreted high levels of anti-inflammatory Th2/Treg cytokines IL-2, IL-4, and IL-10 (Fig. 5E–G). These findings suggest that the athero-protective effect of maternal exposure to a soy protein diet is partly mediated by augmented anti-inflammatory Th2 and regulatory T cell (Treg) responses.

Epigenetic regulation of anti-inflammatory T cell response genes by maternal SPI diet.

As DNA methylation has been implicated in T cell subset generation^{32, 33}, we investigated whether maternal soy protein exposure changes DNA methylation of promoter regions of genes that have been implicated in inflammatory and autoimmune responses (Supplemental Table 3). Of the 24 gene promoter regions evaluated, 5 were hyper-methylated, and 3 were hypo-methylated in CD4⁺ T cells from SPI-offspring compared to CAS-offspring, while 16 of the gene promoter regions did not show any difference in DNA methylation (Supplemental Table 3). Notably, promoter regions of *Gata3* and *Il13* were hypo-methylated, and sphingosine 1-phosphate receptor-3 (*S1pr3*) was hyper-methylated in SPI-offspring compared to CAS-offspring (Supplemental Fig. 4A–C). Levels of mRNA expression of these genes were analyzed to address whether a difference in methylation influences expression. Compared to CAS-offspring, the mRNA expression of *Gata3* and *Il13* were high in CD4⁺ T cells from SPI-offspring (Supplemental Fig. 4D and E). Conversely, *S1pr3* mRNA expression was significantly low in SPI-offspring (Supplemental Fig. 4F). These findings suggest that the epigenetic regulation of genes associated with inflammation could have contributed to attenuated lesions in SPI-offspring.

DISCUSSION

In this study, we completed two objectives. The first objective was to investigate whether maternal exposure to the SPI diet attenuates the progression of atherosclerosis in the adult

offspring of *Apoe*^{-/-} mice, which are hyperlipidemic and develop spontaneous aortic lesions^{28, 34}. The second objective of this study was to delineate the underlying mechanism(s) contributing to the athero-protective effects of maternal exposure to the SPI diet. We showed that maternal exposure to the SPI diet reduced atherosclerotic lesion formation in F1 SPI-offspring, compared with the F1 offspring of CAS-fed *Apoe*^{-/-} dams. Our data also showed that the athero-protective effect of maternal exposure to the SPI diet is partly mediated by decreased inflammatory response and skewed anti-inflammatory macrophage and T cell responses.

Maternal hypercholesterolemia has been implicated in the early onset of fatty streak atherosclerotic lesions in the aortas of fetuses and children²⁻⁵, and F1 offspring of hypercholesterolemic animal models⁶⁻⁹, suggesting that the atherogenic process can start during fetal development. Notably, maternal hypercholesterolemia has been shown to promote transport of maternal cholesterol into fetal circulation^{35, 36}, and to activate endogenous cholesterol in the offspring³⁷. Plasma cholesterol levels of SPI-offspring, however, are indistinguishable from those of CAS-offspring. Moreover, there were no differences in maternal cholesterol levels between CAS- and SPI-fed female mice during mating, or at the time of weaning the pups. These findings suggest that the attenuated lesions in SPI-offspring may be independent of plasma cholesterol levels.

Inflammatory responses contribute to the initiation and progression of atherosclerosis^{11, 12}. Our data show that maternal SPI exposure decreased mRNA and protein expression of pro-inflammatory cytokines in the aorta, and expression of pro-inflammatory cytokines and chemokines in macrophages. These findings suggest that maternal dietary SPI exposure inhibits the progression of hypercholesterolemia-induced atherosclerosis in F1 offspring by potentially downregulating the inflammatory responses (such as VCAM-1, TNF- α , and MCP-1) associated with atherosclerosis^{14, 30, 31}.

An increased presence of IFN- γ -producing CD4+ T cells (Th1 cells) has been shown to promote atherosclerotic lesions^{18, 19}, but anti-inflammatory Th2 (IL-4 and IL-13), and Treg (IL-10) responses are athero-protective³⁸. Our data show that *Ifng* (Th1 cytokine) mRNA expression was lower, and *Il4*, *Il13* (Th2 cytokine), and *Il10* (Treg) expression levels were higher in CD4+ T cells from SPI-offspring compared to those from CAS-offspring. Zhou et al.¹⁷ has shown that *Ifng* expression is high at arterial lesion sites of *Apoe*^{-/-} mice. It has been reported that the interaction of T cells with antigen-presenting cells occurs in the aortic wall³⁹, suggesting that local Th1 response at the lesion site may also contribute to the progression of atherosclerosis. Notably, *Ifng* mRNA expression and IFN- γ secretion by CD4+ T cells were lower, but *Il10* mRNA expression and IL-10 secretion were higher in the aortas of SPI-offspring compared to CAS-offspring.

A system biology approach of peritoneal macrophages isolated from chow-fed or high-fat diet-fed *Ldlr*^{-/-} mice revealed that genes and proteins involved in lipid binding and inflammation are differentially regulated^{40, 41}. Moreover, the genes and proteins identified by this approach are similar to those associated with the progression of atherosclerosis^{40, 41}. In atherosclerotic plaques and the adjacent adventitia, dendritic cells, which are potent antigen-presenting cells, take-up antigens derived from atherosclerotic plaques and migrate

to lymph nodes. These antigens are presented to naïve T cells, resulting in clonal expansion of antigen-specific T cells, and the activated T cells migrate to the lesion site^{12,17–19}. Based on these reports, we used peritoneal macrophages and CD4+ T cells isolated from the spleen to delineate how SPI-mediated anti-inflammatory macrophage and T cell responses contribute to the athero-protective effect of gestational soy exposure. However, in the future, more detailed analyses of macrophages and T cells isolated from the lesion sites and adoptive transfer of T cells from SPI-fed mice are needed to establish the direct relationship to diet-induced anti-inflammatory effects on immune cells including macrophages and T cells.

The idea that DNA methylation⁴² is associated with atherosclerosis has been confirmed both in human atherosclerotic lesions^{43, 44}, and in rabbit and atherosclerosis-prone *apoe*-deficient mice^{45, 46}. Moreover, shear stress and flow-dependent DNA methylation have been shown to regulate endothelial gene expression^{47, 48}, and inhibiting DNA methylation suppresses macrophage inflammatory response⁴⁹. These studies highlight epigenetic mechanisms contributing to atherosclerosis. DNA methylation has been shown to regulate TLR-mediated TNF- α response⁵⁰ and macrophage plasticity⁵¹. Several studies have shown that differentiation of Th1/Th17 and Th2/Treg cells is regulated through DNA methylation of cytokine promoters essential for Th1/Th2 differentiation^{32, 33}. We have demonstrated that DNA methylation induces increased mRNA expression of *Gata3* and *Il13*, transcription factors critical for anti-inflammatory T cell generation, and T cell cytokine response, respectively. We have also demonstrated that promoter regions of these genes are hypomethylated in SPI-offspring. Our findings have also shown that *S1pr3* is hyper-methylated, resulting in reduced *S1pr3* expression. Increased *S1pr3* expression has been shown to increase macrophage recruitment. Furthermore, S1P binding to S1pr3 has been shown to regulate the polarization of T cells to IFN- γ -secreting Th1 cells^{52, 53}. These findings suggest that epigenetic regulation may be associated with increased anti-inflammatory T lymphocyte generation in cases of maternal exposure to soy diet. The soy peptide lunasin and genistein, one of the soy isoflavones, have been shown to induce changes in methylation of CpG islands⁵⁴. Notably, prenatal exposure to genistein alters gene expression and DNA methylation of erythroid precursor cells⁵⁵. DNA methylation changes in cynomolgus monkeys switched from CAS to soy diet suggest that epigenetic changes associated with dietary soy may contribute to the potential health benefits of soy diets⁵⁶. This raises the interesting possibility that maternal exposures to soy diet may negatively regulate pro-inflammatory gene expression by epigenetic regulation, and such a possibility needs to be explored in future studies.

In summary, we have presented evidence that maternal soy diet exposure attenuates the progression of atherosclerosis in the F1 offspring of hypercholesterolemic *Apoe*^{-/-} mice by inhibiting macrophage and T cell inflammatory responses. Collectively, these findings suggest that maternal exposure to the SPI diet inhibits or delays the progression of atherosclerosis in SPI-offspring later in life, by inhibiting inflammatory responses associated with atherogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments.

We thank Mathew Ferguson for his help with the animal experiments. We thank Dr. Thomas Badger and other faculty members at Arkansas Children's Nutrition Center for helpful discussions.

Financial support: This work was supported by a grant from the USDA (CRIS 6251-51000-005-00D) (SN). SN was supported in part by funds from NIH grants (HL086674, HL130126) and Intramural Research Support, Division of Experimental Pathology, Department of Pathology and Vascular Medicine Institute and the Institute for Transfusion Medicine and Hemophilia Center of Western Pennsylvania at the University of Pittsburgh, and Department of Pathology and Laboratory Medicine and UNC Kidney Center, UNC at Chapel Hill, NC (SN).

Abbreviations:

<i>Apoe</i> ^{-/-}	apolipoprotein E knockout
CAS	casein
CVD	cardiovascular disease
MCP-1	monocyte chemoattractant protein-1
PND	postnatal day
SPI	isoflavone-free soy protein isolate
TNF- α	tumor necrosis factor- α
VCAM-1	vascular cell adhesion molecule-1

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Soy Diet Manuscript Key Findings

- Maternal hypercholesterolemia has been implicated in earlier onset of atherosclerotic lesions in neonatal offspring.
- We investigated if **prenatal** exposure to soy diet attenuates the development of atherosclerosis in F1 offspring.
- Pregnant apolipoprotein E knockout female mice were fed SPI diet until postnatal day 21 of the offspring (SPI-offspring).
- While maternal SPI diet did not affect total cholesterol, it did reduce atherosclerotic lesions in SPI-offspring.
- VCAM-1, MCP-1, and TNF-alpha expression are reduced in SPI-offspring.
- In SPI-offspring, expression of pro-inflammatory cytokines by T cells was reduced, and expression of anti-inflammatory cytokines by T cells was augmented.

Fig 1. Maternal SPI exposure attenuates atherosclerotic lesions in *Apoe*^{-/-} offspring.

(A) Experimental study design with dietary regimens is depicted. (B) Body weights were measured weekly and mean body weight gain of the pups at birth until PND140 is represented. Data are mean $\pm$ SD, n=13–14. Aortic sinus (N=7–8/group) were stained using oil red O to visualize plaques and the average lesion area plotted. Aortic sinus from a representative mouse from each group is shown (C, female; E, male mice), and lesion area (D and F) in aortic sinus for all mice (n=7–8) determined. Five slides per mouse were analyzed. The total mean lesion area was analyzed using AxioVision quantitative image analysis software. ** P <0.01 and *** P <0.001 compared to CAS-offspring.

Fig 2. SPI-offspring show reduced VCAM-1 expression in the lesion-prone area.

Vcam1 mRNA expression in the aortas of CAS- and SPI-offspring was determined as described in the Methods section (A). Representative aortic sinus sections from CAS- and SPI-offspring (B) were stained with anti-mouse VCAM-1 IgG to detect VCAM-1 protein expression at the lesion site. Arrows indicate VCAM-1 positive staining. Under similar conditions, aortic sections incubated with an isotype-matched rat IgG control were minimal. VCAM-1 immunopositive areas (C) were quantified in five sections per group, as described in the Methods section. Results are presented as percentage of mean lesion area of CAS-offspring aortas. Values are mean $\pm$ SD from 5 mice. *** $P < 0.001$ compared to CAS-offspring.

Fig 3. Vascular MCP-1 and TNF- α expression is reduced in SPI-offspring.

Mcp1 (A) and *Tnfa* (B) mRNA expression in the aortas of CAS- and SPI-offspring. Each value indicates the mean $\pm$ SD results from five mice. (C) Representative aortic sinus sections from CAS- and SPI-offspring were stained with anti-mouse MCP-1 IgG. Under similar conditions, aortic sections incubated with rat IgG2b control were minimal. Each group contained five mice. (D) MCP-1 immunopositive area was quantified as described in the Methods section. Results are presented as percentage of mean lesion area of CAS-offspring aortas. Values are mean $\pm$ SD from 5 mice. (E) Macrophages in aortic sections from CAS- or SPI-offspring were visualized as described in the Methods section. A representative of five aortic sinus sections from CAS- and SPI-offspring are presented (100 $\times$ magnification). *** p < 0.001 compared to CAS-offspring.

Fig 4. Maternal SPI exposure promotes anti-inflammatory T cell differentiation.

RNA was isolated from purified CD4⁺ cells from CAS- and SPI-offspring (PND140), and mRNA expression of *Ifng* (A), *Il12 p40* (B), *Tnfa* (C), *Il4* (D), *Il10* (E), and *Il13* (F) were determined by qRT-PCR assay. RNA was isolated from the descending aorta, and *Ifng* (G), and *Il10* (H) mRNA expression was determined by qRT-PCR analysis. Each value indicates the mean $\pm$ SD from five mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ compared to CAS-offspring.

Fig 5. CD4 T cells from SPI-offspring show increased anti-inflammatory cytokines.

Purified CD4⁺ T cells from CAS- and SPI-offspring (PND140) were stimulated with plate-bound anti-CD3 and soluble CD28 for 48 h. The concentrations of IFN- γ , TNF- α , IL-6 and IL-17, IL-2, IL-4, and IL-10 (A-G), were measured by cytokine bead array. Each value indicates the mean $\pm$ SD results from four mice.