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# A B-1 Cell Immunization-Based Strategy to Prevent Atherogenesis

Karina Payerhin
Robert M. Berne Cardiovascular Research Center
The University of Virginia
1215 Lee Street
Charlottesville, Virginia 22908 USA

Faculty Advisor: Dr. Coleen McNamara

## Abstract

Cardiovascular disease is a serious health issue responsible for approximately 17.3 million deaths globally each year. Atherosclerosis, the leading contributor to cardiovascular disease, is characterized by the accumulation of low-density lipoprotein (LDL) in blood vessels with subsequent oxidation leading to oxidation specific epitopes (OSE) that are recognized by the body as foreign. Macrophages take up these oxidized lipids and contribute to inflammation, leading to plaque formation. B-1 cells are a subset of B-lymphocytes that produce IgM antibodies against these OSE, preventing oxidized lipid uptake by macrophages. The aim of this project is to determine if increasing the number of B-1 cells and immunizing with oxidized lipids could increase production of IgM antibodies and ameliorate the development and progression of atherosclerosis. To accomplish this, B-1 cells from donor mice were adoptively transferred into atherosclerosis-prone mice ($ApoE^{-/-}$). Half of the mice were immunized with two types of OSE (malondialdehyde-modified LDL (MDA-LDL) and pneumococcal polysaccharide-3 (PPS-3)), and the other half were treated with a control injection. Serum from all mice was collected at five different time points, and levels of IgM to OSE are being measured. The mice were euthanized at 16 weeks after the adoptive transfer, and aortas and aortic roots were collected and will be analyzed for plaque deposition. The study tests the hypothesis that both increasing B-1 cell numbers and immunization with OSE will increase serum IgM to OSE and decrease plaque deposition. These data may lead to the design of an immunization-based approach to treat and prevent human atherosclerosis.

**Keywords: Immunization, atherosclerosis, B-cells**

## 1. Introduction

### 1.1. Atherosclerosis

Cardiovascular disease is a serious health issue responsible for approximately 17.3 million deaths globally per year[1]. Atherosclerosis, the leading contributor to cardiovascular disease, is characterized by the formation of plaque in arteries and blood vessels[2]. This plaque is formed due to the accumulation of low-density lipoprotein (LDL) in arteries and veins. LDL becomes trapped in the intima, which is the innermost layer of an artery or vein, where it becomes oxidized. The oxidation of these particles is mainly stimulated by free radicals and necrotic cell debris in the artery wall[3]. Oxidation results in the formation of oxidation-specific epitopes (OSE) that are recognized by the body as foreign and stimulate an immune response[2]. One of these oxidation adducts is malondialdehyde, or MDA. These particles and other chemoattractant molecules that are produced by the endothelium attract macrophages. Macrophages are involved in atherogenesis due to their ability to take up oxidized lipids like MDA-LDL, resulting in the formation of foam cells, or fat-laden macrophages. The foam cells form fatty streaks in vessel walls, which are the earliest lesions that appear in the formation of atherosclerotic plaque[4]. These lesions are sites of accumulated mononuclear cells, smooth muscle cells, and fibrous tissue that eventually obscure blood flow in the artery[4]. If

untreated, plaque accumulation can lead to heart attack and stroke, both of which can be fatal[5]. Without proper prevention and treatment, cardiovascular disease could cost the global economy up to $47 trillion dollars[1]. Therefore, it is important to investigate the causes of and potential treatments for atherosclerosis.

## 1.2. B-Cell Biology

The body's immune cells are important targets for atherosclerosis treatment. B cells produce antibodies and cytokines, regulate inflammatory responses, and are responsible for presenting various antigens[6]. In both humans and mice, studies identify large numbers of T cells and B cells in early atherosclerotic lesions. In addition, B cell-deficient mice show 30-40% increases in atherosclerotic lesion area in the aorta[7]. Therefore, B cells are thought to be highly involved in the body's immune response to atherosclerosis.

Multiple lineages of B cells exist, each with different characteristics and roles in atherosclerosis. B-2 cells are bone marrow-derived and are found primarily in the spleen and lymph nodes[8]. They contribute to the body's adaptive immune response and are also capable of differentiation into memory B cells[6]. B-2 cells are atherogenic, which is thought to be due in part to the cells' production of proatherogenic IgG and IgE antibodies[2].

In mice, B-1 cells are primarily found in serosal cavities like the peritoneal and pleural cavities. They are fetally derived and undergo self-renewal[2]. There are two known subsets of B-1 cells in mice: B-1a and B-1b cells. B-1a cells have long been known to contribute innate immune responses through their production of circulating natural IgM antibodies[9]. B-1b cells contribute to the body's adaptive and innate immune response by responding to both antigen-dependent and antigen-independent stimuli[10]. Recent research in the McNamara laboratory has established that like B-1a cells, B-1b cells are also capable of producing natural IgM antibodies[10]. An equivalent to murine B-1 cells in humans has been identified, but little is currently known about their function[11].

## 1.3. Role Of B Cells In Atherosclerosis

B-1 cells are important in protection against atherosclerosis due to their production of natural IgM antibodies. These IgM antibodies are specific for oxidation-specific epitopes on oxidized LDL[10]. They bind the oxidized lipids, preventing their uptake by tissue-resident macrophages and the subsequent foam cell formation that leads to atherosclerosis[2]. In addition, natural antibodies have also been shown to facilitate apoptotic cell clearance in atherosclerotic plaques and to limit inflammation, further reducing the development of atherosclerosis[2,12].

The body's immune response to oxidized lipids can be induced in order to reduce the inflammation and plaque formation that characterize atherosclerosis. Studies in mice and rabbits show that immunization of LDL receptor-deficient animals with oxidation epitopes results in a significant reduction of atherosclerosis; a cellular immune response is believed to be responsible for this atheroprotective effect of immunization[13]. Therefore, it is important to examine more closely the role of immune cells and target antibody-producing B-1 cells in particular in an immunization-based strategy for the prevention of atherosclerosis.

## 1.4. Inhibitor Of Differentiation-3

Recent research in Dr. McNamara's laboratory shows that inhibitor of differentiation-3 (Id3), a widely-expressed dominant negative helix-loop-helix (HLH) transcription factor, is important for the development and activity of B cells and their responses to atherosclerotic conditions. Levels of Id3 expression are highest in proliferating cells, and it acts as a differentiation inhibitor in smooth muscle cells, immune cells, and adipocytes[9,14]. Figure 1 details the function of the Id3 protein, which results in the inhibition of DNA binding and transcription[15].

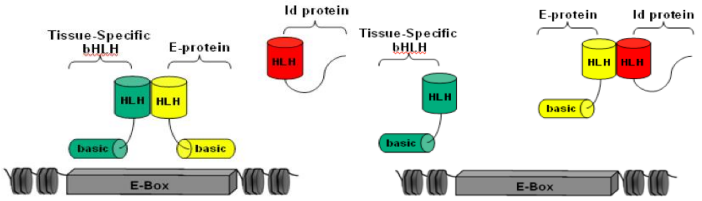


Figure 1. Model of Id3 regulation of DNA transcription

Figure 1. Id3 protein (red) binds to E proteins (yellow), which prevents their dimerization to basic helix-loop-helix (bHLH) dimers (green). DNA binding and transcription are inhibited due to the inability of the bHLH dimer complex to bind to E-boxes and initiate transcription.

Mice lacking Id3 in all cells have significantly increased and early atherosclerosis compared to control mice[5]. These mice develop equal numbers of B-2 cells compared to wild-type mice and reduced numbers of B-1a cells, but increased numbers of B-1b cells[10]. This emphasizes the potentially important role of Id3 in atherosclerosis, as it clearly affects the populations of atheroprotective immune cells.

The loss of Id3 also accounts for reduced B cell homing to the aorta[5]. The few B cells that are present localize to sites of plaque formation in the aorta. These findings establish the role of Id3 as an important player in B cell homing to the aorta under atherosclerotic conditions. Further studies have focused on the role of Id3 specifically in B cells rather than globally in all cells, showing that the transfer of Id3-deficient B cells into B cell-deficient hosts did not provide protection against atherosclerosis whereas wild type B cells were protective, suggesting that Id3 in B cells regulates atheroprotection[5]. Additional findings show that mice with a B cell-specific loss of Id3 ($Id3^{BKO}$) have reduced atherosclerosis, measured by the size of atherosclerotic plaque lesions in the aortic sinus[10]. These mice also showed reduced numbers of lesional apoptotic cells and macrophages as well as an increase in the amount of circulating IgM and T15 antibodies[16]. Furthermore, B-1b cell populations showed a 4-fold expansion in $Id3^{BKO}$ mice compared to control mice[10].

### 1.5. Study Aims

The aim of this study was to determine if increasing the number of B-1 cells and immunizing with antigens mimicking OSE could increase production of IgM antibodies and ameliorate the development and progression of atherosclerosis. To further explore the role of B cells in immunization, B-1a or B-1b cells from wild type or $Id3^{-/-}$ mice were adoptively transferred into atherosclerosis-prone mice ($ApoE^{-/-}$), and half of those mice were immunized with a combination of two antigens mimicking OSE. Serum from all mice was collected at five different time points, and antibody titers were measured by chemiluminescent ELISA. The study tested the hypothesis that both increasing B-1 cell numbers and immunization with antigens mimicking OSE would increase serum IgM to OSE and decrease plaque deposition. These data may lead to the design of an immunization-based approach to treat and prevent human atherosclerosis.

## 2. Methods

### 2.1. Mice

All animal protocols were approved by the Animal Care and Use Committee at the University of Virginia. $Rag1^{-/-}$ mice and $CD19^{cre+}$ mice were provided by Dr. Tim Bender (University of Virginia). $sIgM^{-/-}$ mice were provided by Dr. Peter Lobo (University of Virginia). $Id3^{flox/flox}$ mice were provided by Dr. Yuan Zhang (Duke University). $ApoE^{-/-}$ and C57BL/6 mice were purchased from Jackson Laboratory and bred in house. $Rag1^{-/-}$, $sIgM^{-/-}$, and C57BL/6 mice were used as controls. $CD19^{cre+}$ and $Id3^{flox/flox}$ mice were bred to create $Id3^{-/-}$ mice. $ApoE^{-/-}$ mice were used as atherosclerosis-prone models.

### 2.2. Immunization

The immunization protocol is described in Figure 2. B-1a and B-1b cells were sorted from the peritoneal cavity lavage fluid of CD45.2 $ApoE^{-/-}$ mice, either wild type or $Id3^{-/-}$ (KO). 100,000 B-1a or B-1b cells were injected intraperitoneally into CD45.1 $ApoE^{-/-}$ recipients. CD45 is a protein that was used as a marker to track the injected B cells and distinguish them from existing cells in the mice. One group of mice was immunized with MDA-LDL (100 µg/mouse, LDL from Kalen Biomedical, Montgomery Village, MD, modified with MDA in house) and PPS-3 (pneumococcal polysaccharide 3) (0.5 µg/mouse, ATCC, Manassas, VA), and one group was injected with a control PBS solution. Specific mouse numbers in each group are shown in Figure 3. All mice were bled retro-orbitally at five time points: before B cell transfer, four weeks after B cell transfer/before immunization, 4 weeks after immunization, 8 weeks after immunization, and 13 weeks after immunization. Control serum was collected by retro-orbital blood collection from unimmunized C57BL/6, $ApoE^{-/-}$, $sIgM^{-/-}$, and $Rag1^{-/-}$ mice.

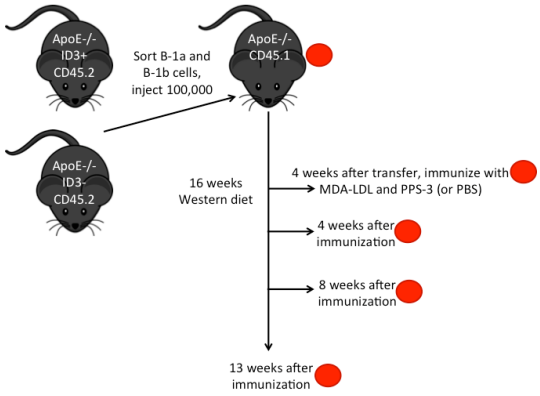


Figure 2. Immunization strategy

Figure 2. B-1a and B-1b cells were sorted and transferred into recipient mice. Half of the mice were immunized with malondialdehyde-modified LDL (MDA-LDL) and pneumococcal polysaccharide-3 (PPS-3), and the other half were treated with a control injection. Mice were bled and serum was collected before B cell transfer, 4 weeks after transfer, 4 weeks after immunization, 8 weeks after immunization, and 13 weeks after immunization.

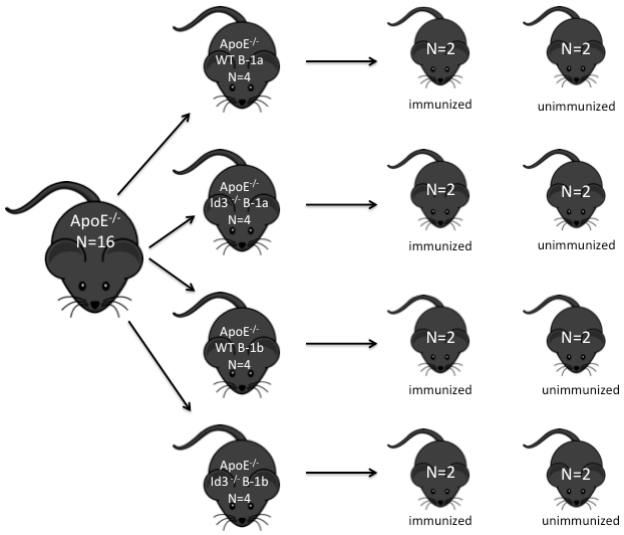


Figure 3. Sample size and mouse numbers

Figure 3. All 16 mice that received B cells in this experiment were CD45.1$^{+}$ ApoE$^{-/-}$. Of these mice, 4 groups of 4 mice each received different populations of B cells by adoptive transfer: recipients of wild type B-1a cells, Id3$^{-/-}$ B-1a cells, wild type B-1b cells, or Id3$^{-/-}$ B-1b cells. Within each of these groups of B cell recipients, 2 mice were immunized with MDA-LDL and PPS-3, and 2 were injected with a control PBS solution.

## 2.3. Chemiluminescent ELISA

Specific antibody levels in mouse serum were measured by chemiluminescent ELISA. The assays were performed using white, 96-well immunoplates (Thermo Fisher Scientific, Waltham MA). All capture and detection antibodies and standards were from Southern Biotech (Birmingham, AL). Plates were coated overnight at 4°C with the following capture antigens or antibodies (100 µl/well): MDA-LDL (5 µg/ml), anti-IgM (0.625 µg/ml), anti-IgG1 (0.625 µg/ml), and anti-IgG2c (2.5 µg/ml) in coating buffer (containing 0.1 disodium phosphate in deionized water, pH 9.0). The plates were washed three times with washing buffer (PBS with 0.1% Tween 20) using an automated plate washer and were then blocked with BSA/PBS (150 µl/well) at room temperature for 2 hours. Serum samples were diluted in BSA-

PBS at the following concentrations: 1:1000 for IgM to MDA-LDL, 1:50,000 for total IgM, 1:10,000 for IgG1, and 1:50,000 for IgG2c. These concentrations were chosen after serum samples pooled from all mice were tested at various dilutions against a standard curve. BSA-PBS was added to blank wells (100 μl/well). The samples were incubated for 48 hours at 4°C and then washed three times.

The plates were then incubated with detection antibodies conjugated to alkaline phosphatase (100 μl/well) at the following concentrations: anti-IgM-AP (1:4000), anti-IgG1-AP (1:8000), and anti-IgG2c-AP (1:4000). After six washes with washing buffer, plates were washed once with deionized water before reading. The amount of antibody bound was measured using chemiluminescent technique: 50 ml of LumiPhos 530 solution (Lumigen, Southfield, MI) was added to each well, and luminescence was determined using the FLUOstar Optima microplate reader (BMG Labtech, Ortenberg, Germany). Data were calculated either as microgram per milliliter, based on standard curves of isotype standards, or expressed as relative light units counted per 100 milliseconds (RLUs/100 ms) where quantitative standards were not commercially available.

## 3. Results

### 3.1. Control

Figure 4 shows data from a control plate that was tested to determine the functionality of the assay. The levels of IgM to MDA-LDL were tested by chemiluminescent ELISA in serum from unimmunized mice of the genotype ApoE$^{-/-}$ and normal C57BL/6 (B6) at a dilution of 1:1000, and Rag1$^{-/-}$ and sIgM$^{-/-}$ at a dilution of both 1:1000 and 1:100. Rag1$^{-/-}$ mice have no B or T cells, while sIgM$^{-/-}$ mice are incapable of secreting IgM, so these mice served as controls since it was expected that they would not secrete any IgM. The data show that ApoE$^{-/-}$ mice have significantly higher levels of IgM against MDA-LDL, which is likely due to already high levels of lipids in their serum since they are atherosclerosis-prone models, which characterizes the disease. Therefore, their serum levels of natural antibodies against MDA-LDL are expected to be much higher than in normal mice, as measured, in response to the elevated lipid levels already present in the blood.

As expected, B6 mice had much lower levels of IgM antibody production because the levels of lipids in their serum are lower. Rag1$^{-/-}$ and sIgM$^{-/-}$ mice showed no measurable levels of IgM to MDA-LDL, even at concentrations 10 times higher (1:100) than those tested for the immunocompetent mice, which is expected for mouse models that do not have functioning immune cells.

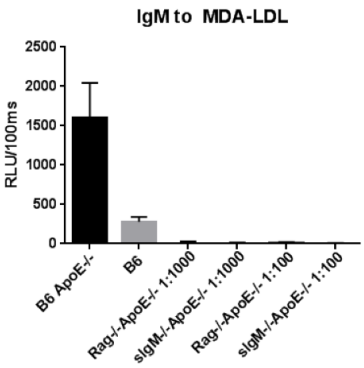


Figure 4. Levels of serum IgM to MDA-LDL in unimmunized mice

Figure 4. Levels of serum IgM to MDA-LDL in unimmunized mice, measured in relative light units per 100 milliseconds (RLU/100 ms). Serum from ApoE$^{-/-}$ and B6 mice was measured at a dilution of 1:1000, and serum from Rag1$^{-/-}$ and sIgM$^{-/-}$ mice was measured at dilutions of both 1:1000 and 1:100. Data are presented as the mean ± SEM. N=3 for each group of mice.

### 3.2. IgM to MDA-LDL

Figure 5 shows levels of IgM to MDA-LDL displayed as fold increases from pre-immunization to 4 weeks, 8 weeks,

and 13 weeks post-immunization. After 13 weeks, the fold increase in levels of IgM to MDA-LDL was nearly the same in all unimmunized mice, regardless of whether B-1a or B-1b cells were initially injected. In immunized mice, recipients of both B-1a and B-1b cells from Id3$^{-/-}$ donors showed an increase in IgM levels after immunization, with one recipient of B-1a cells nearly doubling by the end of the experiment (13 weeks). However, immunized mice that received B cells from wild type donors maintained similar levels of IgM, despite the immunization. These results suggest that the loss of Id3 in B cells could stimulate the production of IgM antibodies to MDA-LDL as a result of immunization, while immunization does not seem to have an effect on the production of natural IgM antibodies by wild type B cells.

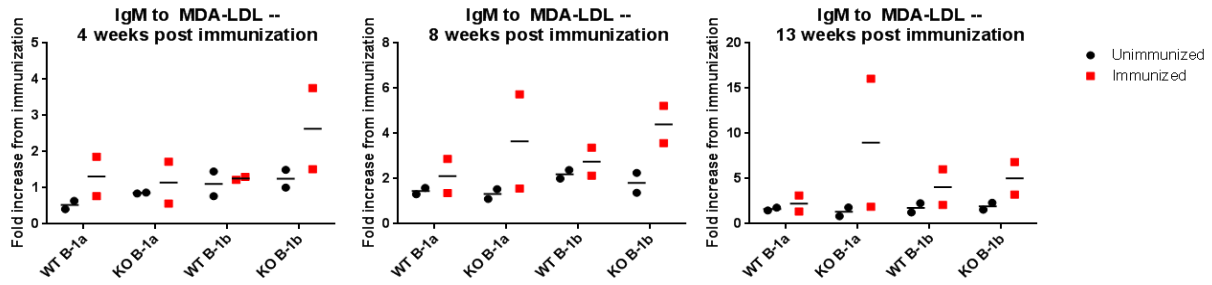


Figure 5. Levels of serum IgM to MDA-LDL after immunization

Figure 5. Levels of serum IgM to MDA-LDL at 4 weeks, 8 weeks, and 13 weeks after immunization, represented as a fold change when compared to levels before immunization.

## 3.3. Total IgM

All unimmunized mice showed a steady fold increase in levels of total IgM during the course of the study, but there were no differences among the mouse genotypes or the types of B cell received in the transfer (Fig. 6). A slight fold increase in total IgM levels is seen over time in immunized mice that received wild type B-1a cells, and levels in mice that received wild type B-1b cells increased with immunization even more noticeably. Immunized recipients of B-1a cells from Id3$^{-/-}$ donors showed a slight increase in IgM levels during the experiment, with recipients of B-1b cells showing a smaller increase after immunization. These data suggest a potential positive effect of immunization on the production of IgM antibodies, independent of Id3. In addition, a greater fold increase in mice injected with B-1b cells supports previous findings that showed significantly higher production of IgM antibodies in B-1b cells compared to B-1a cells[10]. Again, this may be due to more production of IgM by B-1b cells than by B-1a cells, or because B-1b cells have better cell viability after transfer.

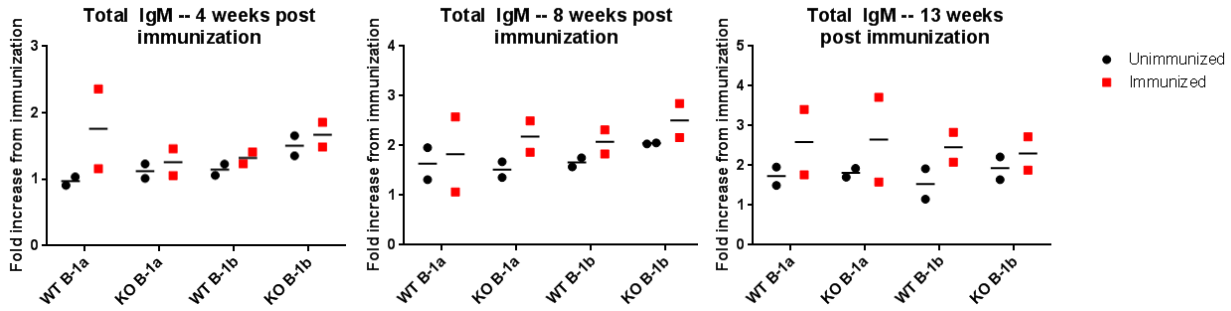


Figure 6. Levels of total serum IgM after immunization

Figure 6. Levels of total serum IgM at 4 weeks, 8 weeks, and 13 weeks after immunization, represented as a fold change when compared to levels before immunization.

## 3.4. IgG1

Immunized mice that received wild type B-1a cells showed a significant fold increase in IgG1 levels, but no difference was observed in unimmunized mice (Fig. 7). Mice receiving wild type B-1b cells showed a slight fold increase after immunization. The data also show a marked difference in fold increase for unimmunized mice compared to immunized mice receiving B-1a cells from Id3$^{-/-}$ mice at 8 weeks, but by the end of the experiment, the fold increase is similar. In contrast, recipients of Id3$^{-/-}$ B-1b cells showed a similar fold increase until the end of the experiment, where immunized mice showed a slightly greater fold increase in levels of IgG1 than unimmunized mice. These data suggest that wild type B-1a cells had an effect on the production of IgG1 after immunization, which is interesting considering that B cells primarily produce IgM antibodies.

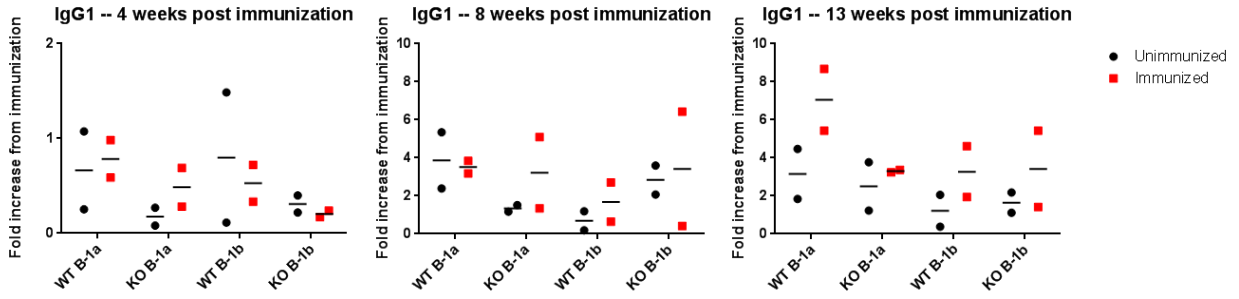


Figure 7. Levels of serum IgG1 after immunization

Figure 7. Levels of serum IgG1 at 4 weeks, 8 weeks, and 13 weeks after immunization, represented as a fold change when compared to levels before immunization.

## 3.5. IgG2c

Figure 8 shows a fold decrease in levels of IgG2c in all mice after 13 weeks, regardless of immunization or B cell transfer. The immunized mice that received wild type B-1a cells showed the greatest decrease, nearly threefold by the end of the experiment. There was no difference in the fold change between immunized and unimmunized mice that received wild type B-1b cells, Id3$^{-/-}$ B-1a cells, or Id3$^{-/-}$ B-1b cells. This fold decrease in all cell types, despite immunization, could be connected to increased production of other antibodies after immunization. It is possible that not as much IgG2c was produced due to the increased levels of IgM antibodies. Again, since B-1 cells produce primarily IgM antibodies, it is not surprising that no fold increase in IgG2c was seen. It is interesting that there was such a significant fold decrease, but this may have been due to more IgM production and therefore no compensation was seen in the levels of IgG2c antibodies.

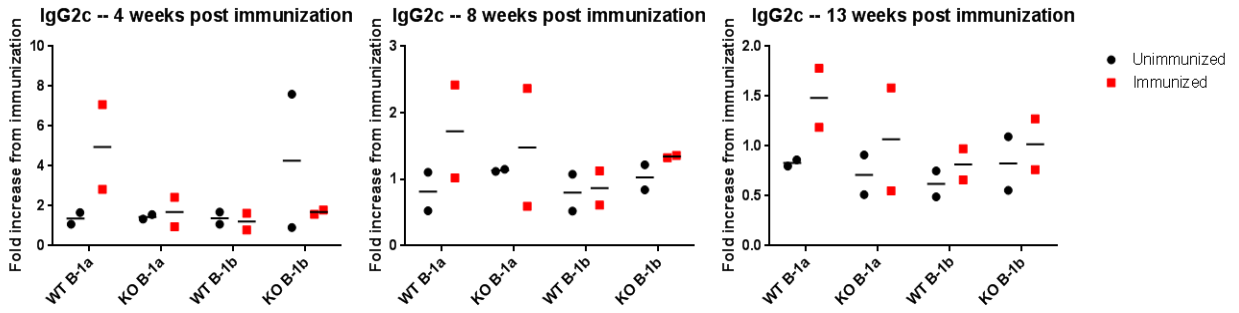


Figure 8. Levels of serum IgG2c after immunization

Figure 8. Levels of serum IgG2c at 4 weeks, 8 weeks, and 13 weeks after immunization, represented as a fold change when compared to levels before immunization.

## 4. Discussion and Conclusions

### 4.1. Discussion

This study revealed limited conclusive information about the specific effects of immunization, largely due to the small sample size in the study, but it established the feasibility of the approach and identified several potential trends to be tested in follow-up studies with larger cohorts. Most notably, B cells lacking Id3 produce more IgM antibodies against MDA-LDL after immunization. Previous studies show an expansion in B cell populations due to the loss of Id3 specifically in B cells ($Id3^{BKO}$)[10]. Perhaps the increased antibody production by these B cells is the result of an increase in the overall B cell population, adding to the stimulation of antibody production by immunization (Fig. 5).

The data also show an overall positive trend in the effects of immunization on the total levels of IgM in serum, especially in mice injected with B-1b cells, but this effect is Id3-independent. In contrast, the trend seen in levels of IgM to MDA-LDL seems to be dependent on Id3. This may suggest that immunization with oxidized lipids stimulates the production of natural antibodies by B cells regardless of conditions or genotype, while the loss of Id3 combined with immunization results in a more specific response to MDA-LDL. Other antibodies tested, including IgG1 and IgG2c, showed no notable effects as expected, consistent with data that the primary antibody produced by the B-1 cells injected in this experiment was $IgM^2$.

The sample size was a limiting factor in this study. The McNamara lab breeds all of its own mice, so not enough sex-matched and age-matched mice (all male, 13-15 weeks old) were available at the time of this experiment to be used for the entire duration of the study (13 weeks). The study split the mice into eight different groups to allow for the investigation of all combinations of B cell types and immunization (Fig. 3). Another result of the small sample size was the large variation in levels between two mice in certain groups, affecting the calculation of the average fold increase since neither mouse could be considered an outlier. In addition, statistical analysis could not be performed on these data, but several potential trends are seen and can be confirmed with a larger sample size.

### 4.2. Conclusions And Future Directions

The study aimed to test the hypothesis that an increase in B-1 cell numbers by adoptive transfer and immunization with antigens that mimic OSE on LDL would increase the production of atheroprotective natural antibodies. The results could be used to design an immunization-based approach for the treatment and prevention of human atherosclerosis. Due to a small sample size, it is difficult to draw definitive conclusions from the data, but this study validated the feasibility of the study, established a working ELISA to evaluate the effects, and demonstrated a positive trend that suggests a potential positive effect of immunization with antigens that mimic OSE on LDL in mice, which can be applicable to humans.

This study should be repeated with a larger sample size to account for mice that might be outliers and so that statistics can be performed on the data. In addition, a cell-based therapy could be explored in which B cells would be activated with antigens that mimic OSE on LDL *in vitro* before being transferred into recipient mice to ensure their capability of producing antibodies. Finally, it would be useful to conduct a comparison of the survival of B-1a and B-1b cells to investigate whether the differences in their antibody production is due to one subset actually producing more antibodies from equal numbers of cells, or if one subset simply survives better *in vivo* after transfer than the other.

## 5. Acknowledgements

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