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Immune-modulation by polyclonal IgM treatment reduces atherosclerosis in hypercholesterolemic apoE−/− mice

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ABSTRACT

Objective: Gamma-globulin treatment reduces experimental atherosclerosis by modulating immune function; however the effect of IgM on atherosclerosis is not known. We investigated the effect of serum-derived, non-immune polyclonal IgM (Poly-IgM) on atherosclerosis in mice with advanced disease and also assessed its immune-modulatory effects.

Methods and results: Aortic atherosclerosis was assessed in apoE−/− mice fed atherogenic diet starting at 6 weeks of age. In addition, mice were also subjected to perivascular cuff injury to the carotid artery at 25 weeks of age to induce accelerated atherosclerosis. At the time of injury, the mice were treated weekly with a commercially available Poly-IgM (0.4 mg/mouse) or PBS for 4 weeks and euthanized at 29 weeks of age. Poly-IgM reduced aortic atherosclerosis, and reduced lesion size in the aortic sinus and injured carotid artery, without significant changes in serum cholesterol levels. Poly-IgM treatment was associated with increased anti-oxLDL IgG titers and a reduction in the % splenic $CD4^+$ T cells compared to controls. The splenic $CD4^+$ T cell cultured from the Poly-IgM treated mice had reduced proliferation in vitro compared with controls.

Conclusion: Poly-IgM treatment reduced aortic and accelerated carotid atherosclerosis in apoE−/− mice in association with increased anti-oxLDL IgG titers, and reduced number and proliferative function of splenic $CD4^+$ T cells. Our study identifies a novel athero-protective and immunomodulatory role for non-immune polyclonal IgM.

1. Introduction

Atherosclerosis is a complex disease characterized by an inflammatory response that is modulated by the immune system [1,2]. Evidence supporting the role of the immune response to the disease has accumulated such that modulation of the immune response is now viewed as a potential avenue of therapy [3]. Intravenous immunoglobulin (ivIg) is used to modulate immune function in various auto-immune diseases. Its potential therapeutic use in atherosclerosis has been demonstrated in animal models of both spontaneous atherosclerotic disease and accelerated lesion formation [4,5]. The mechanism of action involves down-modulation of unfavorable immune function, including reduction of inflammatory cytokines [5]. Other known effects of ivIg treatment include enhancement of the auto-antibody repertoire and down-regulation of T cell function [4]. Recent reports have suggested that the immune-regulatory role of immunoglobulin treatment may not be limited to the IgG fraction. IgM may have similar properties that result in down-regulation of the inflammatory response [6–8]. Furthermore, we recently reported that non-immune, serum-derived polyclonal IgM antibodies were just as effective as IgG antibodies in reducing neointimal formation after arterial injury. The results we reported were independent of their modulatory effect on the immune system since the study was conducted using immune-deficient Rag-1−/− mice [9]. We sought to extend the findings by using serum-derived polyclonal IgM in hypercholesterolemic apoE−/− mice. The effect of serum-derived polyclonal IgM on both spontaneous atherosclerosis and accelerated lesion formation were tested and potential immune-regulatory properties were investigated. Our results provide evidence that polyclonal IgM reduces atherosclerosis and modulates immune function by enhancing the auto-antibody repertoire and down-regulating number and proliferation of $CD4^+$ T cells.

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Table 1
Effect of polyclonal IgM treatment on atherosclerosis.

	% plaque area (*en face*)	Aortic sinus lesion area (mm^2)	Carotid artery lesion area (mm^2)
PBS	21.01 ± 5.05 (n = 15)	0.64 ± 0.11 (n = 8)	0.034 ± 0.025 (n = 12)
Poly-IgM	15.86 ± 6.35 (n = 12)*	0.45 ± 0.12 (n = 8)†	0.015 ± 0.010 (n = 12)*

Poly-IgM: polyclonal IgM
* $p < 0.05$ vs. PBS.
† $p < 0.01$ vs. PBS.

2. Methods

2.1. Mice

Male apoE−/− mice on the C57Bl/6J background were purchased from Jackson Laboratories (Bar Harbor, ME) at 6 weeks of age and were fed Western type diet (0.2% cholesterol, 20% fat, TD88137, Harlan Teklad) throughout the duration of the experiment. At the age of 25 weeks, a non-occlusive plastic cuff (length = 2.5 mm, internal diameter = 0.51 mm; Cole-Parmer Instrument Co.) was aseptically placed around the right carotid artery and the skin incision was closed as previously described [10]. The mice then received intra-peritoneal injections of serum-derived mouse polyclonal IgM (Poly-IgM, Rockland) at a dose of 0.4 mg/mouse, or PBS once a week until euthanasia four weeks later. A hybridoma cell line secreting a monoclonal anti-phosphorylcholine IgM with the T15 idiotype (HPCM2 cells, a generous gift from Dr. Patricia Gearhart at NIH/NIA) was maintained [11] as a source for injection of anti-phosphorylcholine IgM to another group of mice also at a dose of 0.4 mg/mouse until euthanasia four weeks later. The dose was chosen based on a previous report that after injection of 0.4 mg IgM the mean level of IgM in Rag1−/− mouse was similar to that of normal mouse [12].

At euthanasia, blood was collected and the aorta and carotid arteries were perfused with 0.9% saline for 10 min. Harvested carotid arteries were embedded in OCT compound (Tissue-Tek, Allegiance), and frozen at −80 °C. The collected spleens were either flash-frozen or processed for flow cytometric analysis and cell culture. Aortas were collected for en face atherosclerotic lesion staining with Oil-Red-O [13]. All procedures were approved by the Institutional Animal Care and Use Committee.

2.2. Morphometric measurement and immunohistochemistry

Frozen sections (6–8 μm thick) were collected from the injured right carotid arteries and contra-lateral uninjured left carotid arteries. Four sections were collected on each slide and 20–25 slides were collected from each injured arterial segment, as described previously [14]. Slides were stained with hematoxylin and eosin, and the vessel area measured using computer assisted morphometric analysis (Image-Pro Plus). The measurements of sections from each animal were averaged for analysis.

For monocyte/macrophage staining, sections were fixed in ice-cold acetone for 5 min and standard immunohistochemical protocol was performed using monocyte/macrophage antibody (MOMA-2, Serotec). Omission of primary antibody was used as negative control.

For Oil Red O staining, frozen sections were fixed in 4% paraformaldehyde at room temperature for 30 min. Slides were stained with 0.24% Oil Red O in 60% isopropanol for 20 min and counterstained with hematoxylin and fast-green. The stain area was measured using computer assisted morphometric analysis.

En face preparations of the aortas were also visualized and analyzed using computer assisted morphometric analysis.

2.3. ELISA

Antibody titer to oxLDL was assessed using standard ELISA technique. Briefly, plates were coated with 10 μg/ml Cu-oxLDL (Biomedical Technologies) as capture antigen. Sera were diluted (1:100 for IgG or 1:50 for IgM titers) and incubated on coated plates for 2 h in 4 °C, washed and detected using HRP labeled anti mouse IgG (Pierce) or anti mouse IgM with the ABTS system (SBA). Values are expressed as OD 405. Total serum IgM levels were measured using a commercially available kit (Biosource).

2.4. Serum cholesterol

Serum cholesterol levels were measured using commercially available kits (Sigma; Wako).

2.5. Flow cytometry

After RBC lysis, splenocytes were stained with fluorescent labeled antibodies for CD4, CD8b, and CD28 (eBiotech) and subjected to flow cytometric analysis (BD Bioscience). Binding of polyclonal IgM to T cells was tested using FITC-labeled Poly-IgM and PE-labeled CD4 or CD8b antibodies. FITC-labeled poly IgM was produced in the laboratory using labeling kit from Pierce.

2.6. Splenocyte proliferation

Splenocytes were labeled with CFSE (2.5 mM, Molecular Probes) for 10 min in 37 °C, washed, and cultured in RPMI media with 10% FBS. Cells were then treated with Concanavalin-A (Con-A, 3 μg/ml) and collected 5 days later. Cells were stained with PE-labeled CD4 or CD8b antibody for flow cytometric analysis. CFSE staining intensity was analyzed using lymphocytes gated on either CD4 or CD8b. Analysis was performed by determining the percentage of cells that had reduced CFSE intensity, indicating cell proliferation.

2.7. Statistical analysis

Results are presented as mean ± SD. Student's t-test was used to test for significance, set at $p < 0.05$.

3. Results

3.1. Polyclonal IgM reduces atherosclerosis

Serum IgM levels were measurably increased in the Poly-IgM treated mice compared with PBS treated mice at euthanasia and were comparable between the two groups (128 ± 4 vs. 119 ± 3 μg/ml, respectively; $p = 0.08$). Treatment with Poly-IgM significantly reduced *en-face* aortic atherosclerosis compared with PBS treatment (Fig. 1A and B; Table 1). Plaque area in the aortic sinus was significantly reduced by Poly-IgM treatment (Fig. 1C and D; Table 1). Accelerated lesion formation induced by cuffing of the right carotid artery was also reduced by Poly-IgM treatment (Fig. 1E and F; Table 1). No lesions were present in the contra-lateral left carotid arteries (Fig. 1G and H). No significant changes were

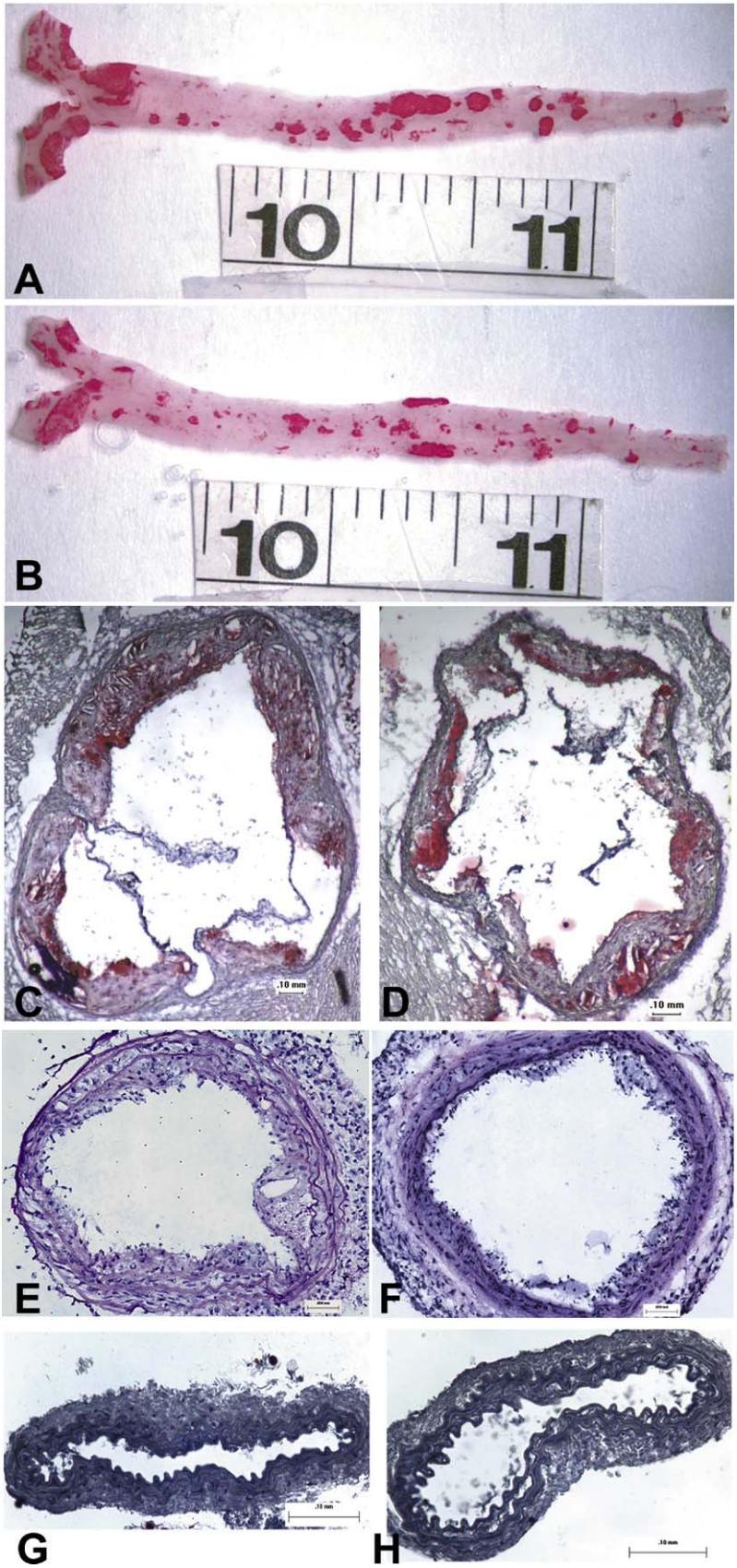

Fig. 1. Representative photograph of *en face* staining of atherosclerotic plaques in the aorta of PBS treated control (A) and polyclonal IgM treated (B) mice. Oil Red O stain of aortic sinus plaques from PBS treated (C) and polyclonal IgM treated mice (D). Bar = 100 μm. Hematoxylin and eosin stain of 28-day injured right carotid artery from PBS treated (E) and polyclonal IgM treated (F) mice. Bar = 50 μm. Contra-lateral uninjured left carotid artery from PBS treated (G) and polyclonal IgM treated (H) mice. Bar = 100 μm.

Table 2
Serum cholesterol profile.

	Total cholesterol	LDL	HDL	Triglyceride
PBS	812 ± 358	404 ± 48	42 ± 28	30 ± 14
Poly-IgM	999 ± 563	367 ± 131	31 ± 21	48 ± 43

All values are in mg/dl.

observed in macrophage or lipid content (not shown) in the aortic sinus and right carotid artery lesion, and serum cholesterol levels were comparable between the two groups (Table 2).

3.2. Alteration of the natural antibody repertoire by polyclonal IgM

The immune modulatory effect of intravenous immunoglobulin treatment is attributed to several mechanisms, including enhancement of the natural antibody repertoire and down-regulation of T cell function. The first mechanism was tested by determining relative anti-oxLDL antibody titers in the experimental groups. oxLDL IgG was significantly increased in the Poly-IgM group compared with PBS group (Fig. 2A). There was a significant correlation between anti-oxLDL IgG and *en-face* aortic atherosclerosis in the Poly-IgM treated group ($p = 0.05$; Fig. 2B) but not in the PBS treated group ($p = 0.4$).

There was no significant difference in anti-oxLDL IgM titers between the two groups (Fig. 2C). However, as in the case of anti-oxLDL IgG titers, there was a significant correlation between anti-oxLDL IgM titers and *en-face* aortic atherosclerosis in the Poly-IgM treated group ($p = 0.015$; Fig. 2D) but not in the PBS treated group ($p = 0.7$).

3.3. Down-regulation of T cells by Poly-IgM treatment

To determine the T cell population to which Poly-IgM binds, flow cytometry was performed on spleen cells from non-treated apoE−/− mice using PE-labeled CD4 or CD8b antibodies and FITC-labeled Poly-IgM or PBS. The results showed that Poly-IgM has the ability to bind to both $CD4^+$ T cells and $CD8^+$ T cells (Fig. 3A and B).

Flow cytometry of $CD4^+$ T cells from spleens of Poly-IgM treated mice showed a significant reduction in their number compared with mice treated with PBS (Fig. 4A). This was not observed in $CD8b^+$ T cells (Fig. 4B). $CD4^+CD28^+$ T cells were also significantly reduced in Poly-IgM treated mice compared with mice treated with PBS (Fig. 4C). There was no difference in $CD8b^+CD28^+$ T cells between the two groups (Fig. 4D). To further determine if $CD4^+$ T cells from Poly-IgM treated mice had reduced response to an immunogenic challenge, splenocytes from Poly-IgM and PBS treated mice were cultured after staining with CFSE and treated with Con-A to determine proliferative activity (Fig. 4E, inset). $CD4^+$ splenocytes from Poly-IgM treated mice proliferated significantly less than $CD4^+$ splenocytes from PBS treated mice (Fig. 4E). Proliferation of $CD8b^+$ splenocytes was not significantly different between the two groups (Fig. 4F).

3.4. Atherosclerosis is not reduced by a mouse monoclonal IgM antibody

To determine if the effect of Poly-IgM was specific for polyreactive natural antibodies, we treated another group of mice with the mouse monoclonal anti-phosphorylcholine IgM antibody (PC-IgM) which we previously demonstrated to reduce syngeneic vein graft atherosclerosis [11]. Treatment with PC-IgM did not reduce atherosclerosis in the aorta ($17.25 \pm 7.00\%$; $n = 12$) or in the cuffed carotid artery ($0.057 \pm 0.049\,mm^2$; $n = 6$). Anti-oxLDL IgG titers were also unchanged by PC-IgM treatment (not shown), indicating that there was no effect on the natural antibody repertoire.

4. Discussion

The results of our study show reduced spontaneous atherosclerosis in the aorta and decreased accelerated lesion formation in the cuffed carotid artery by polyclonal IgM treatment in hypercholesterolemic apoE−/− mice. It should be noted that these favorable results were observed despite a relatively short duration of treatment (4 weeks) which was initiated at 25 weeks of age when mice were expected to already have advanced atherosclerotic lesions. This is noteworthy since many experimental therapies have been tested prior to, or at the earlier stages in the evolution of atherosclerotic disease [4,5,13,14]. Indeed, we have previously shown that timing affects the efficacy of immunization for atherosclerosis [13]. The current study shows that polyclonal IgM was effective in reducing advanced spontaneous aortic atherosclerosis as well as injury accelerated carotid atherosclerosis.

The serum IgM levels in the Poly-IgM treated mice were elevated but did not reach statistical significance most likely because the serum sample were taken at euthanasia, which is one week after the last Poly-IgM injection. We have previously reported the kinetics of absorption of injected IgM in mice [11].

Immunoglobulin therapy is known to have multiple effects on immune function, including enhancing the auto-antibody repertoire and down-modulating T cell function. We first tested the auto-antibody repertoire to oxLDL. IgG and IgM antibodies to oxLDL are known to occur spontaneously, and we have previously shown that the titers increase with age [15]. Our results show that polyclonal IgM treatment resulted in increased anti-oxLDL IgG antibodies. Anti-oxLDL IgG antibody titers were inversely correlated with *en-face* aortic atherosclerosis in the polyclonal IgM treated group, but not in the PBS group. In addition, although there was no difference in anti-oxLDL IgM titers between the two groups, an inverse correlation between anti-oxLDL IgM titers and *en-face* aortic atherosclerosis, was observed in the polyclonal IgM treated group but not in the PBS group. The results show that the anti-oxLDL antibody repertoire was modified by polyclonal IgM treatment in association with reduced atherosclerotic plaque area. This is in contrast to the report by Nicoletti et al. wherein treatment of apoE−/− mice with intravenous IgG did not affect anti-oxLDL IgG titers, but did reduce anti-oxLDL IgM titers [4]. Thus, it appears that the effects of intravenous IgG on the auto-antibody repertoire are different from those of polyclonal IgM observed in our study. Interestingly, LDLR−/− mice deficient in soluble IgM had significantly reduced anti-oxLDL IgG titers associated with increased atherosclerosis [16]. Thus, IgM may have yet undefined functions that modulate the auto-antibody repertoire in atherosclerosis.

Nicoletti et al. also showed that the proliferative capacity of T cells collected from the lymph nodes was inhibited by intravenous IgG treatment [4]. However, the subtype of T cells affected was not identified in that study. We show that $CD4^+$ T cells are preferentially reduced in the spleen of polyclonal IgM treated mice, with no significant effect on $CD8b^+$ T cells. The pathogenic role of $CD4^+$ T cells in atherosclerosis is well described [17] and the specific reduction likely contributed to the effect on plaque growth. We further tested the proliferative capacity of both cell types after stimulation with Con-A. $CD4^+$ T cells from polyclonal IgM treated mice showed significantly reduced proliferation than $CD4^+$ T cells from PBS treated mice. Consistent with the in vivo result, proliferation of $CD8^+$ T cells was not significantly affected by polyclonal IgM treatment.

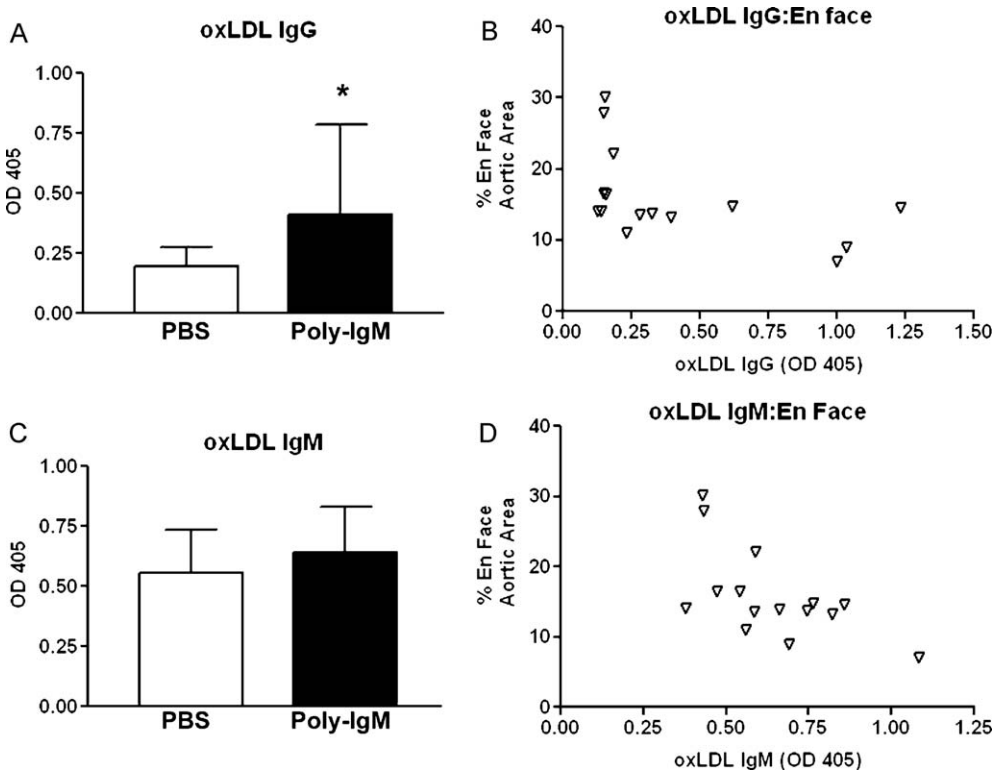

Fig. 2. Increased oxLDL IgG antibody titers in polyclonal IgM treated mice compared (Poly-IgM) with PBS treated control mice (A). $^{*}p < 0.05$. Negative correlation was found between anti-oxLDL IgG titers and *en-face* aortic atherosclerosis in the polyclonal IgM treated mice (B). Anti-oxLDL IgM antibody titers were not affected by polyclonal IgM treatment (C), but were negatively correlated with *en-face* aortic atherosclerosis (D).

IgM-enriched intravenous Ig has been shown to decrease expression of the T cell activation marker CD69 in a humanized SCID model [8]. In our study, $CD4^{+}CD28^{+}$ T cells from Poly-IgM treated mice were significantly reduced compared to PBS. Our results are in agreement with their observation that IgM, like intravenous IgG also regulates T cell function. However, it remains unclear how Poly-IgM treatment regulates T cell function. IgM purified from serum contains anti-leukocyte antibodies (IgM-ALA) that immunoprecipitated lymphocyte markers such as CD3 and CD4, and have been shown to inhibit T cell function [18]. Our results showing binding of FITC labeled Poly-IgM to a small percentage of T cells and alteration of $CD4^{+}$ T cell function is consistent with their report.

We recently reported that natural antibodies of the IgG and IgM isotypes reduced neointimal formation in the immune-deficient Rag-1−/− mice [9]. The current report is in agreement with our previous work on the inhibitory effect of IgM on vascular lesion formation. However, because the Rag-1−/− mice have no mature immune cells, the effects were likely mediated through a different mechanism.

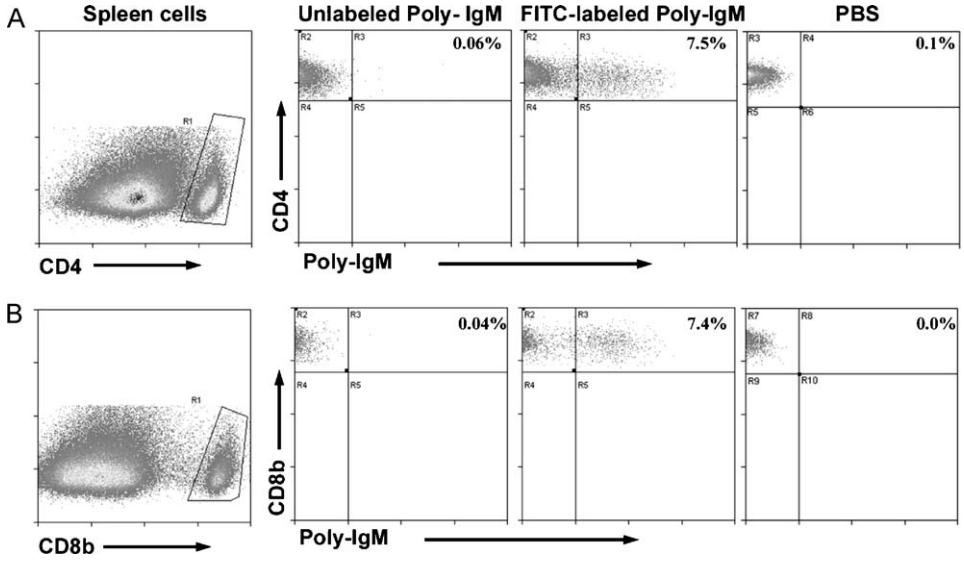

Fig. 3. FITC-labeled polyclonal IgM (Poly-IgM) was used to stain PE-labeled CD4 (A) or CD8 (B) cells to assess Poly-IgM binding to T cells. Control for staining was unlabeled polyclonal IgM or PBS.

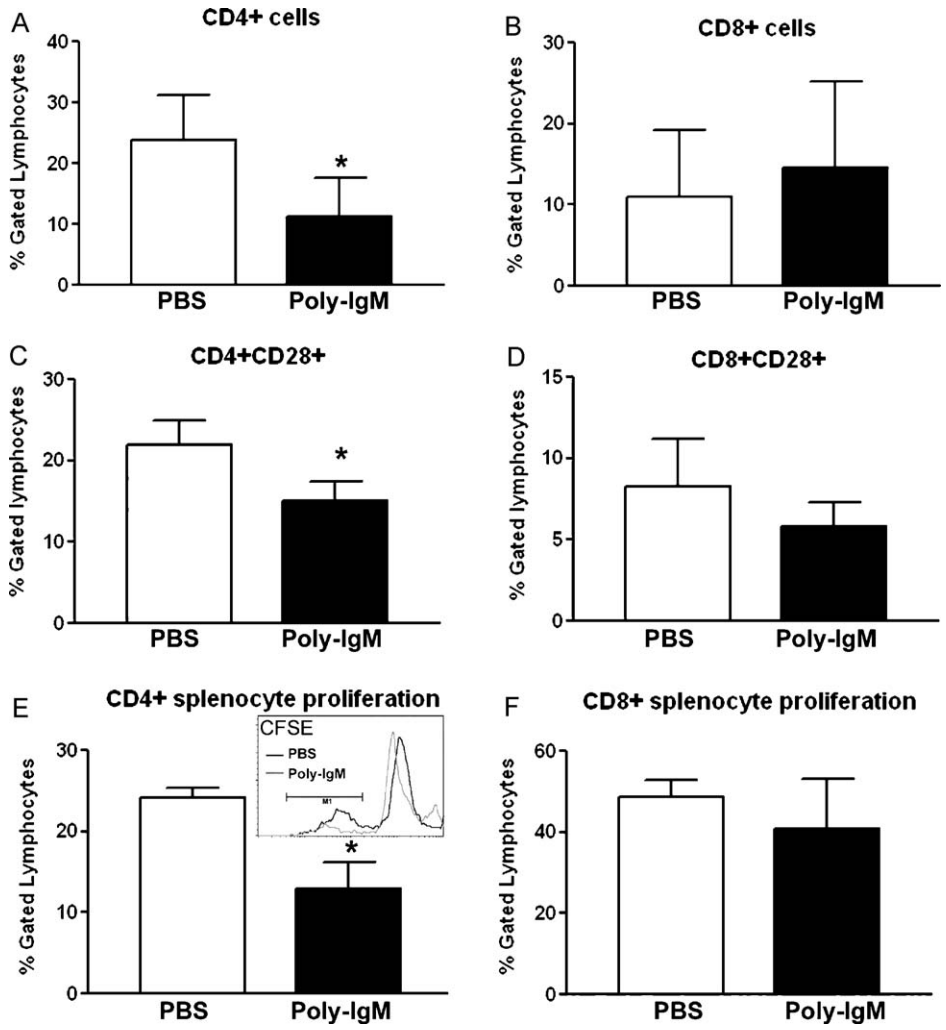

Fig. 4. Percentage of $CD4^+$ cells from the spleen of mice treated with polyclonal IgM were significantly reduced compared with PBS treated control mice (A). No significant effect was observed in $CD8b^+$ cells (B). Polyclonal IgM treatment significantly reduced the percentage of $CD4^+CD28^+$ T cells in the spleen compared to PBS treated group (C). No significant difference was observed in the $CD8b^+CD28^+$ T cells in the spleen (D). Proliferation of CFSE labeled, $CD4^+$ gated splenocytes, stimulated with 3 μg/ml of Concanavalin A, was reduced by polyclonal IgM treatment (E). Inset (E) shows CFSE profile of PBS treated control (black) and polyclonal IgM treated splenocytes (grey). No significant difference was observed in $CD8b^+$ splenocytes (F). $^*p < 0.05$; $n = 4$ each.

In conclusion, our study shows that polyclonal IgM reduces advanced spontaneous aortic atherosclerosis as well as injury-induced accelerated carotid atherosclerosis in apoE−/− mice. Polyclonal IgM treatment significantly enhanced the auto-antibody repertoire to oxLDL and preferentially reduced the number and proliferative function of splenic $CD4^+$ T cells without significantly affecting $CD8^+$ T cells. Polyclonal IgM may be an alternative therapeutic approach to modulate the immune response to atherosclerotic disease.

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Disclosures

The authors have no financial interests to disclose.

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