

Anti-TIM-1 Monoclonal Antibody (RMT1-10) Attenuates Atherosclerosis By Expanding IgM-producing B1a Cells

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Background—Peritoneal B1a cells attenuate atherosclerosis by secreting natural polyclonal immunoglobulin M (IgM). Regulatory B cells expressing T-cell immunoglobulin mucin domain-1 (TIM-1) expanded through TIM-1 ligation by anti-TIM-1 monoclonal antibody (RMT1-10) induces immune tolerance.

Methods and Results—We examined the capacity of RMT1-10 to expand peritoneal B1a cells to prevent atherosclerosis development and retard progression of established atherosclerosis. RMT1-10 treatment selectively doubled peritoneal B1a cells, tripled TIM-1⁺ B1a cells and increased TIM-1⁺IgM⁺interleukin (IL)-10⁺ by 3-fold and TIM-1⁺IgM⁺IL-10⁻ B1a cells by 2.5-fold. Similar expansion of B1a B cells was observed in spleens. These effects reduced atherosclerotic lesion size, increased plasma IgM and lesion IgM deposits, and decreased oxidatively modified low-density lipoproteins in lesions. Lesion CD4⁺ and CD8⁺ T cells, macrophages and monocyte chemoattractant protein-1, vascular cell adhesion molecule-1, expression of proinflammatory cytokines monocyte chemoattractant protein-1, vascular cell adhesion molecule-1, IL1 β , apoptotic cell numbers and necrotic cores were also reduced. RMT1-10 treatment failed to expand peritoneal B1a cells and reduce atherosclerosis after splenectomy that reduces B1a cells, indicating that these effects are B1a cell-dependent. Apolipoprotein E-KO mice fed a high-fat diet for 6 weeks before treatment with RMT1-10 also increased TIM-1⁺IgM⁺IL-10⁺ and TIM-1⁺IgM⁺IL-10⁻ B1a cells and IgM levels and attenuated progression of established atherosclerosis.

Conclusions—RMT1-10 treatment attenuates atherosclerosis development and progression by selectively expanding IgM producing atheroprotective B1a cells. Antibody-based in vivo expansion of B1a cells could be an attractive approach for treating atherosclerosis. (*J Am Heart Assoc.* 2018;7:e008447. DOI: 10.1161/JAHA.117.008447.)

Key Words: atherosclerosis • B1a cells • IgM • immune system • RMT1-10 • TIM-1

We have previously reported that peritoneal B1a cells are atheroprotective¹ and proposed that therapeutic expansion of atheroprotective B1a cells might be an approach to reduce atherosclerosis.² Their atheroprotective effect is associated with B1a-derived polyclonal natural immunoglobulin M (IgM) which accumulates in atherosclerotic lesions to reduce apoptotic cell numbers and necrotic core size, thereby

reducing the severity of inflammation in lesions; IgM plays a key role in recognizing phosphatidylserine on apoptotic cells, facilitating apoptotic cell removal by phagocytosis.³ B1a cells have been classified as a subset of regulatory B cells.⁴ A regulatory CD1d^{hi}CD5⁺ B cell that produces interleukin (IL)-10 controls T cell-dependent inflammatory responses.⁵ Regulatory B cells producing IL-10 have also been shown to suppress inflammatory disorders including arthritis, allergy, ulcerative colitis, and experimental autoimmune encephalomyelitis.^{6–8} IL10, a potent anti-atherogenic cytokine,⁹ is critical for sustaining the expansion of CD5⁺ B cells.¹⁰ Regulatory B cells have been identified in humans.¹¹

TIM-1 is a member of the TIM family of cell surface phosphatidylserine receptors in humans and in mice¹² that directly couples to phosphotyrosine-dependent intracellular signaling pathways¹³ and provides a costimulatory signal for T cell activation.¹³ TIM-1 has recently been found to be predominantly expressed on regulatory B cells.¹⁴ Ligation of TIM-1 by a low affinity anti-TIM-1 monoclonal antibody (RMT1-10) promotes immune tolerance via IL-10-expressing B cells.¹⁴ Treatment also increases TIM-1⁺ B cell numbers

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Clinical Perspective

What Is New?

- Antagonist T-cell immunoglobulin mucin domain-1 (clone RMT1-10) treatment reduces atherosclerosis development and retards progression of established atherosclerosis via selectively expanding immunoglobulin M producing B1a cells in animal models.

What Are the Clinical Implications?

- Antibody-mediated in-vivo expansion of atheroprotective B1a cells may be a promising therapeutic approach to attenuate progression of established atherosclerosis.
- This therapeutic strategy may be attractive to treat patients with atherosclerosis to prevent atherosclerosis-related vascular disorders such as heart attack and stroke.

and the percentage of TIM-1+ B cells expressing IL-10 and IL-4,¹⁴ preserving regulatory T cells but inhibiting CD4+ Th1 cell expansion.¹⁵ TIM-1 has been identified in human regulatory B cells.¹⁶

In the present study, we sought to investigate whether RMT1-10 treatment also expands the IgM producing B1a cell population and is effective in attenuating atherosclerosis. We show that 40% of B1a cells in the peritoneal cavity also express TIM-1 and that treatment with RMT1-10 markedly increases the number of TIM-1⁺ peritoneal B1a cells expressing IgM with or without IL-10. The expansion of B1a cells attenuates both atherosclerosis development and progression of developed atherosclerosis, associated with increased plasma IgM and its deposits in lesions and decreased oxidatively modified low-density lipoproteins (oxLDLs) in lesions. This effect of RMT1-10 treatment on atherosclerosis is dependent on B1a cells because it is not seen in B1a cell-depleted splenectomized mice.

Methods

The data, analytical methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure.

Animal Ethics, Atherogenic Diet, and Tissue Collection

All experimental procedures and study protocols complied with the Guide of Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85-23, revised 2011) were approved by the Animal Ethics Committee of the Alfred Medical, Research, and Education Precinct (AMREP), Prahran, Melbourne, Australia, and animal

experiments were conducted at the Precinct Animal Centre, AMREP where Apolipoprotein E (ApoE)-KO mice on C57BL/6 background were bred and maintained. Male ApoE-KO mice on C57BL/6 background (6–8 week-old) were fed a high-fat diet (HFD) containing 21% fat and 0.15% cholesterol (Specialty Feeds, Glen Forrest, Western Australia). In this study, only male mice were chosen as in the majority of previous studies carried out by us and by others. At the end of experiments, mice were culled, and spleens and peritoneal fluids were collected for differential cell analysis by flow cytometry; OCT-embedded aortic roots, snap-frozen aortic arches, and plasma were kept in a –80°C freezer for subsequent analysis.

TIM-1 Monoclonal Antibody Treatment and Experimental Protocol

Rat anti-mouse TIM-1 IgG2a RMT1-10 (clone RMT1-10, BioXcell) was used to stimulate TIM-1 receptor and controlled with an isotype-matched control rat anti-mouse IgG2a Ab (Sigma).^{14,15} In the prevention study, ApoE-KO mice received either RMT1-10 or IgG control intraperitoneally (0.2 mg/mouse)^{14,17} on alternate days during an 8-week HFD. In the intervention study,^{14,17} ApoE-KO mice fed an HFD for 6 weeks to establish atherosclerosis were treated with the same regimen of either RMT1-10 or control IgG2a antibody while continuing an HFD for 8 weeks.

B1a Cells Depletion Using Splenectomy

In splenectomy experiments, spleens of 6- to 8-week-old ApoE-KO mice were removed surgically under aseptic conditions to remove peritoneal B1a cells as described previously.^{1,18,19} In sham operation, spleen was treated as the way in splenectomy procedure without removing the spleen.¹

Plasma Lipid Profiles

Plasma concentration of total cholesterol, high-density lipoprotein cholesterol, very-low-density lipoprotein/LDL (VLDL/LDL) cholesterol, and triglycerides were determined enzymatically using a cholesterol assay kit (Roche/Hitachi) and automated chemistry analyzer.²⁰

Flow Cytometry

B lymphocytes and non-B-lymphocyte populations in spleen and peritoneal cavity were analyzed with fluorochrome conjugated antibodies (BD Pharmingen, San Diego, CA) on FACS-Canto II (BD Biosciences) as described.^{1,20,21} Phycoerythrin (PE)-conjugated anti-CD19, Allophycocyanin (APC)-conjugated anti-CD5, APC-Cy7-conjugated anti-CD11b, Pacific Blue-

conjugated anti-CD4, PerCP-conjugated anti-CD8 α , Fluorescein isothiocyanate (FITC)-conjugated anti-TCR-b and PE-Cy7-conjugated anti-NK1.1 antibodies were used in lymphocyte analysis. In intracellular staining, cells were stimulated for 5 to 6 hours with Cell Stimulation Cocktail plus Protein Transport Inhibitors (eBioscience, San Diego, CA). After blocking Fc receptors, surface markers were first stained with PE-conjugated anti-TIM1 (clone RMT1-4), APC-conjugated anti-CD5, FITC-conjugated anti-CD19, PE-Cy7-conjugated anti-CD1d and PerCP-conjugated anti-IgM antibodies. Fixed and permeabilized cells were stained with Pacific Blue-conjugated anti-IL-10 antibody. To analyze Th1/Th2 cells, splenocytes were stimulated and stained as described using APC-conjugated anti-TCR β , APC-Cy7-conjugated anti-CD4, PE-Cy7-conjugated anti-IL-4, Alexa Fluor 488-conjugated anti-IL-6, PE-conjugated anti-tumor necrosis factor- α , PerCP-Cy5.5-conjugated anti-interferon gamma, Pacific Blue-conjugated anti-IL-10 antibodies. Data acquired on FACS-Canto II (BD Biosciences) were analyzed using FACS-Diva software (BD Biosciences).

Histological Lesion Analysis At Aortic Roots

Atherosclerosis develops in aortic roots and other vascular beds in hyperlipidemic ApoE-KO mice, and aortic roots are most common regions for atherosclerosis assessment²² and atherosclerosis assessed at aortic roots correlate highly with those measured in the entire aorta.²³ In this study, we used aortic roots to quantify atherosclerotic lesions and aortic arches for differential mRNA expression. In accordance with the American Heart Association statement^{22,24} frozen sections (6 μ m) were cut from the aortic sinus, the region where the valve or valve cusps first become visible to where the left and right coronary arteries branch off.²⁵ To assess atherosclerosis, Oil Red O-stained atherosclerotic lesions at aortic sinus (6 sequential sections at 80- μ m intervals) were used to measure both total intimal lesion areas and Oil Red O-stained lipid deposition areas as described before.²⁰ For necrotic core assessment in atherosclerotic lesions, aortic root atherosclerotic lesions were stained with hematoxylin and eosin (H&E) to identify acellular areas as necrotic cores and measured as described previously.^{1,26,27} To determine lesion efferocytosis, macrophages in atherosclerotic lesions were double-labeled with CD68 and terminal dUTP nick end-labeling (TUNEL) using immunofluorescence. Both macrophage-associated and free TUNEL-positive apoptotic cells were quantified using Olympus BX61 fluorescence microscope and images captured using FV11 Olympus.

Immunohistochemical Analysis At Aortic Roots

CD68+ macrophage, CD4+ and CD8+ T cell accumulation, oxLDL antigen, IgM and IgG deposition and IL-1 β , MCP-1, and

VCAM-1 protein expression at aortic root atherosclerotic lesions were assessed by immunohistochemical analyses as described.^{21,28} Apoptotic cells identified by terminal dUTP nick end-labeling (TUNEL) under light microscopy were expressed per lesion areas as described before.^{20,28}

ELISA

ELISA was used to determine plasma levels of total and oxLDL-specific antibodies (Ig, IgG, and IgM levels) as described before^{1,29} and plasma IgM levels of anti-CD3 and anti-CD4 antibodies as described before.³⁰ Plasma IL-10 levels were determined by an ELISA kit (eBioscience) as recommended by the manufacturer.

Arterial RNA Extraction and mRNA Expression Analysis

RNeasy fibrous tissue mini kit (Qiagen) was used to extract total RNA from aortic arches and single-step QuantiFast SYBR Green reverse transcription-polymerase chain reaction (RT-PCR) kit (Qiagen) was used to measure mRNA expression in genes of interest as described before.^{21,27,28}

Statistical Analyses

Results are expressed as means \pm SEM. Comparisons between groups were carried out using Student t test or Mann-Whitney *U* test, depending on whether the data were normally distributed, as assessed using the Kolmogorov-Smirnov test. For multiple comparisons, results were analyzed using one-way ANOVA (after confirming normality of distribution) followed by Bonferroni post-test. A value of $P < 0.05$ was considered statistically significant.

Results

RMT1-10 Treatment Expands B1a Cells

Previous studies using RMT1-10 treatment have been limited to short-term treatment.¹⁴ We used a prolonged therapeutic strategy involving administration of RMT1-10 every other day for 8 weeks whilst ApoE-KO mice were fed an HFD. RMT1-10 treatment doubled the number of peritoneal B1a cells ($P < 0.05$; Figures 1A and 1B) and whilst B1a cells in spleen tended to increase, this was not statistically significant ($P > 0.05$; Figure 1B). RMT1-10 treatment increased TIM-1 expression on peritoneal B1a cells from 40% to 62% and together with increased peritoneal B1a cells (Figures 1A and 1B), treated mice showed increased peritoneal B1a cells by nearly 3-fold ($P < 0.05$; Figure 1C); a similar trend of increased B1a cells in the spleen did not reach statistical significance

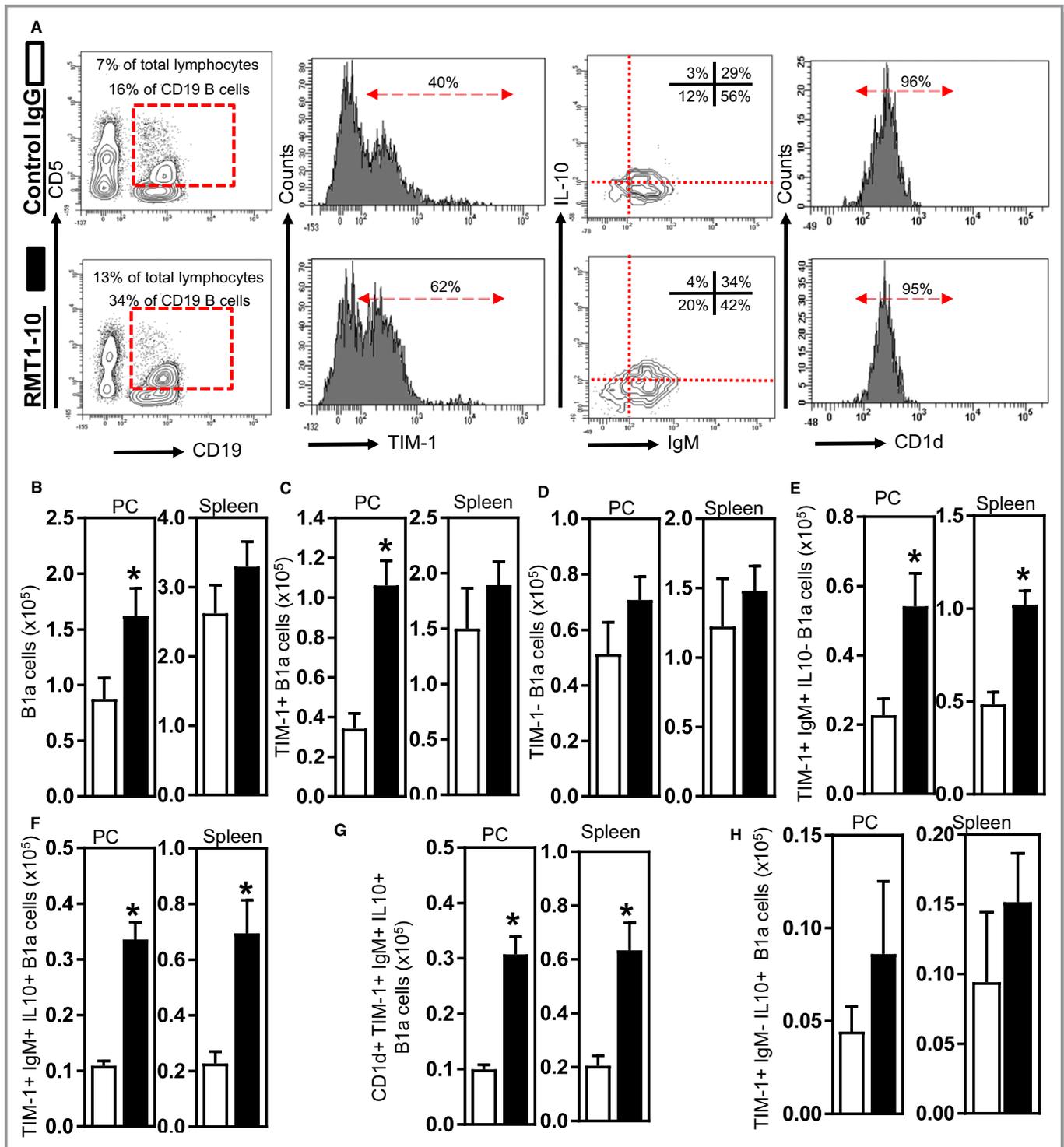


Figure 1. B1a cells and B1a cells subclasses expand following anti-TIM-1 (RMT1-10) antibody treatment. ApoE^{-/-} mice were treated with RMT1-10 antibody at the beginning of an 8-week high fat diet. A, Representative flow cytometry plots showed increased expression of TIM-1, IgM, IL-10 ad CD1d on PC B1a cells in treated mice. RMT1-10 treatment increased (B) CD19+CD5+ B1a cells, (C) TIM-1⁺ B1a cells without affecting (D) TIM-1⁻ B1a cells. It also increased (E) TIM-1⁺IgM⁺L-10⁻, (F) TIM-1⁺IgM⁺IL-10⁺ and (G) CD1d+TIM-1+IgM+IL10+ B1a cells in the spleen and peritoneal cavity. H, TIM-1⁺IgM⁻L-10⁺ B1a cells were unaffected by RMT1-10 treatment. Data represent mean±SEM, *P<0.05, unpaired t test, n=13 in control (control IgG-treated) and n=16 in test (RMT1-10-treated) groups. IgM indicates immunoglobulin M; IL10, interleukin-10; PC, peritoneal cavity; TIM-1, T-cell immunoglobulin and mucin domain-1.

(Figure 1C). Peritoneal and spleen TIM-1- B1a cells did not change their numbers after RMT1-10 treatment (Figure 1D) consistent with a TIM-1-mediated mechanism in their expansion. The numbers of TIM-1+ B1a cells expressing IgM alone (Figure 1A) increased ≈ 2.5 -fold and 2-fold in the peritoneum and spleen, respectively, ($P < 0.05$; Figure 1E). TIM-1+ IgM+ IL-10+ B1a cells were similarly increased 3-fold in the peritoneal

cavity and spleen ($P < 0.05$; Figure 1F). Majority of TIM-1+ IgM+ IL-10+ B1a cells express CD1d (Figure 1A) and RMT1-10 treatment also increased numbers of CD1d-expressing TIM-1+ IgM+ IL-10+ B1a cells (Figure 1G) as well as regulatory B cell as defined by CD19+ CD5+ CD1d+, majority of which modulate immune responses by IL-10³¹ (Figure 2A). In contrast, TIM-1+ IgM- IL-10+ B1a cells were unaffected by

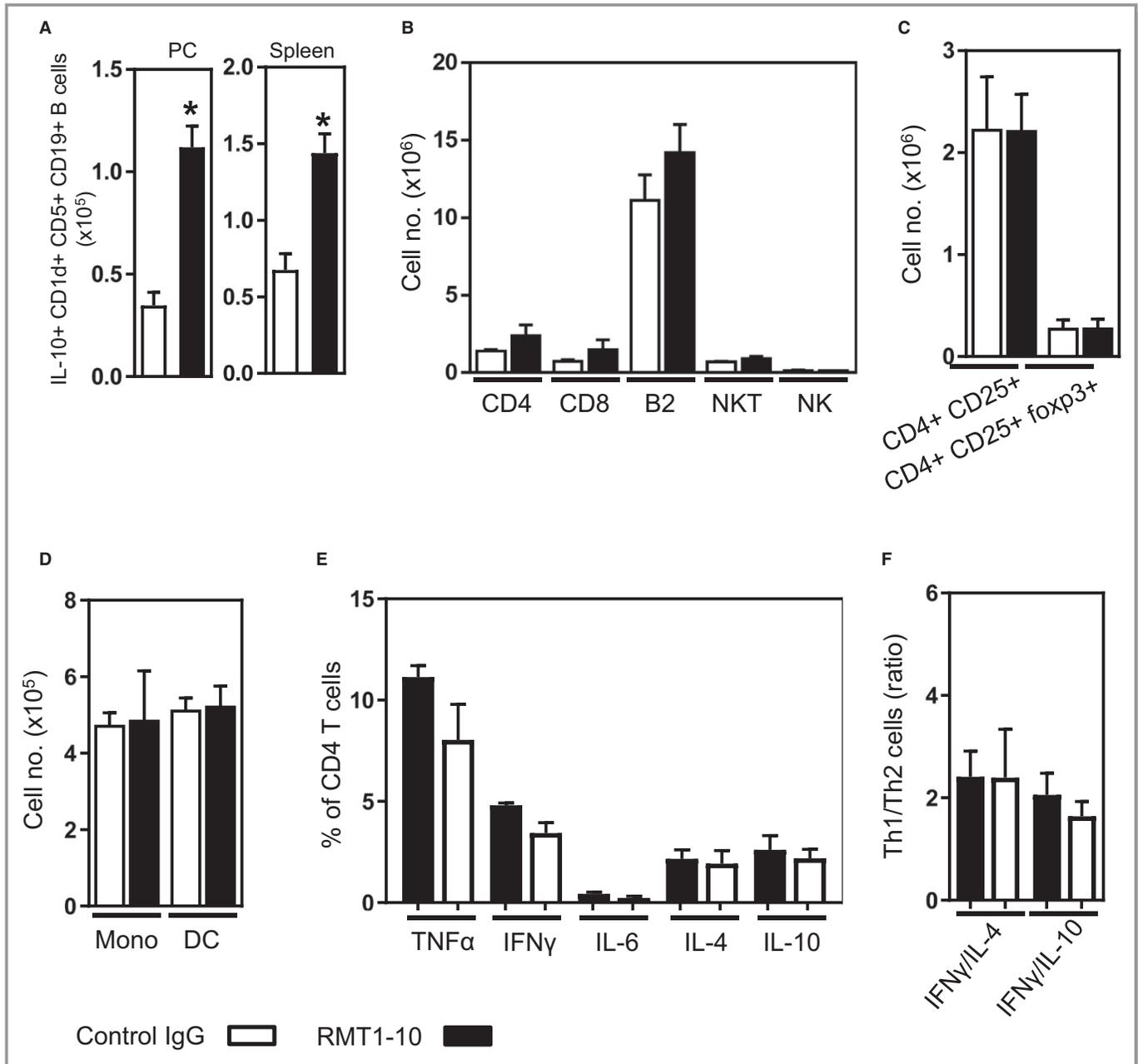


Figure 2. RMT1-10 treatment increases regulatory B cells without affecting other immune cells. ApoE^{-/-} mice were treated with RMT1-10 antibody at the beginning of 8-week high fat diet and different immune cells in spleens were analyzed at the end of experiment. A, CD1d+CD5+CD19+ regulatory B cells were increased in the spleen and peritoneal cavity, however (B) lymphocytes, (C) regulatory T cells, (D) monocytes and dendritic cells, (E) Th1 and Th2 cells as well as (F) ratio of Th1/Th2 cells were unaffected by RMT1-10 treatment. Data represent mean \pm SEM, unpaired t test. n=13 in control (control IgG-treated) and n=16 in test (RMT1-10-treated) groups. B2 indicates B2 B cells; CD4, CD4 T cells; CD8, CD8 T cells; DC, dendritic cells; IFN, Interferon; IL, interleukin; Mono, monocyte; NK, Natural killer cells; NKT, Natural killer T cells; PC, peritoneal cavity; TNF, tumor necrosis factor. * $P < 0.05$

RMT1-10 treatment (Figure 1H) indicating the ability of RMT1-10 to expand TIM-1⁺ IgM⁺ B1a cells specifically. Other immune cells including monocytes, dendritic cells, regulatory T cells and Th1/Th2 T cell ratio in spleens were unaffected (Figure 2).

RMT1-10 Treatment Increases Plasma IgM Levels and Atherosclerotic Deposits of IgM

We next examined if RMT1-10 treatment elevated B1a-derived IgM levels. Consistent with the increase in B1a cell numbers, plasma levels of total IgM and Malondialdehyde (MDA)-oxLDL-specific IgM were increased by 33% and 40%, respectively, by RMT1-10 treatment ($P<0.05$; Figure 3A). In contrast, plasma total Ig and IgG levels were unaffected, as were MDA-oxLDL-Ig and IgG levels (data not shown). Furthermore, plasma levels of anti-CD3 and anti-CD4 -specific IgM antibodies were also elevated by 78% and 100%, respectively, in RMT1-10 treated mice ($P<0.05$; Figure 3A). As lesion IgM facilitates removal of apoptotic cells³² and prevents accumulation of oxLDL in lesions,¹ we examined whether RMT1-10 treatment affected lesion IgM and oxLDL. RMT1-10 treatment increased lesion IgM levels by $\approx 170\%$ ($P<0.05$; Figure 3B). MDA-oxLDL accumulation in lesions was reduced by nearly 30% ($P<0.05$; Figure 3C). Plasma IL-10 determined by ELISA were not affected by RMT1-10 treatment (Figure 4A).

RMT1-10 Treatment Reduces Atherosclerotic Lesions, Apoptotic Cells, and Necrotic Core Size

We next investigated the effect of expanded B1a cells and increased IgM following RMT1-10 treatment on atherosclerosis development. Chronic RMT1-10 treatment of hyperlipidemic ApoE-KO mice attenuated atherosclerosis with 50% reduction in total lesion size. Lipid and macrophage accumulation were also markedly reduced, by 40% and 38%, respectively, (all $P<0.05$; Figures 5A and 5B), however the ratio of lipid and macrophage accumulation was not affected (Figure 4B). Because IgM facilitates removal of apoptotic cells,^{33,34} we assessed the effects of RMT1-10 treatment on lesion apoptotic cell numbers. Treatment with RMT1-10 reduced apoptotic cell numbers in lesions by 30% ($P<0.05$; Figure 5C). As apoptotic cell numbers are related to the size of the necrotic core,³⁵ we next assessed necrotic core size. Necrotic core in lesions of RMT1-10-treated mice were reduced by nearly 50% ($P<0.05$; Figure 5D). Accumulated apoptotic cells and/or apoptotic bodies are cleared via phagocyte-mediated efferocytosis and defective efferocytosis by macrophages in atherosclerotic lesions contribute to

necrotic core generation.³⁶ Therefore, we investigated the effect of RMT1-10 treatment on lesion efferocytosis and found that efferocytosis improved by 81% in mice treated with TIM-1 mAb (Figure 5E). RMT1-10 treatment did not affect plasma lipids or body weights (Figures 5F and 5G).

RMT1-10 Treatment Reduces Arterial Inflammation

To determine whether RMT1-10 treatment reduced inflammation in developing lesions, we next assessed its effects on lesion T cell numbers and expression of proinflammatory proteins and cytokines. Immunohistochemistry was performed to assess effects on T cells as well as expression of monocyte chemoattractant protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1). RMT1-10 treatment reduced CD4⁺ T cell numbers in lesions by 50% and CD8⁺ T cell numbers by 35% ($P<0.05$; Figures 6A and 6B). Also, expression of MCP-1 was reduced by nearly 40% in lesions ($P<0.05$; Figure 6C), as was expression of VCAM-1, by 55% ($P<0.05$; Figure 6D); mRNA encoding MCP-1 and VCAM-1 were also reduced, by 43% and 40%, respectively, ($P<0.05$; Figure 6E). RMT1-10 treatment also reduced lesion mRNA expression of proinflammatory cytokine, IL-1 β by $\approx 50\%$ ($P<0.05$; Figure 6E).

Anti-Atherosclerotic Effect of RMT1-10 Therapy is Dependent on B1a Cells

Since TIM-1 can also be expressed on other immune cells, albeit at much lower levels.¹⁴ We next assessed the effects of RMT1-10 on atherosclerosis development in splenectomized ApoE-KO mice because B1a cells can be selectively depleted by splenectomy.^{1,18} At the end of the study we confirmed the splenectomy-related depletion of B1a cells in the peritoneal cavity (Figure 7A). B1a cells in the peritoneal cavity were reduced by $\approx 75\%$, averaging 1.45×10^4 cells compared with 6.5×10^4 cells in non-splenectomized mice ($P<0.05$; Figure 5B), consistent with previous studies.^{1,18} RMT1-10 treatment did not increase peritoneal B1a cells nor reduced atherosclerotic lesions in splenectomized ApoE-KO mice (Figures 7B and 7C).

Anti-TIM-1 Therapy Attenuates Progression of Developed Atherosclerosis

To determine therapeutic potential of RMT1-10 treatment on atherosclerosis, ApoE-KO mice were fed an HFD for 6 weeks after which they were treated with RMT1-10 for an additional 8 weeks of HFD. RMT1-10 treatment selectively increased the number of B1a cells by 66% in the peritoneal cavity ($P<0.05$;

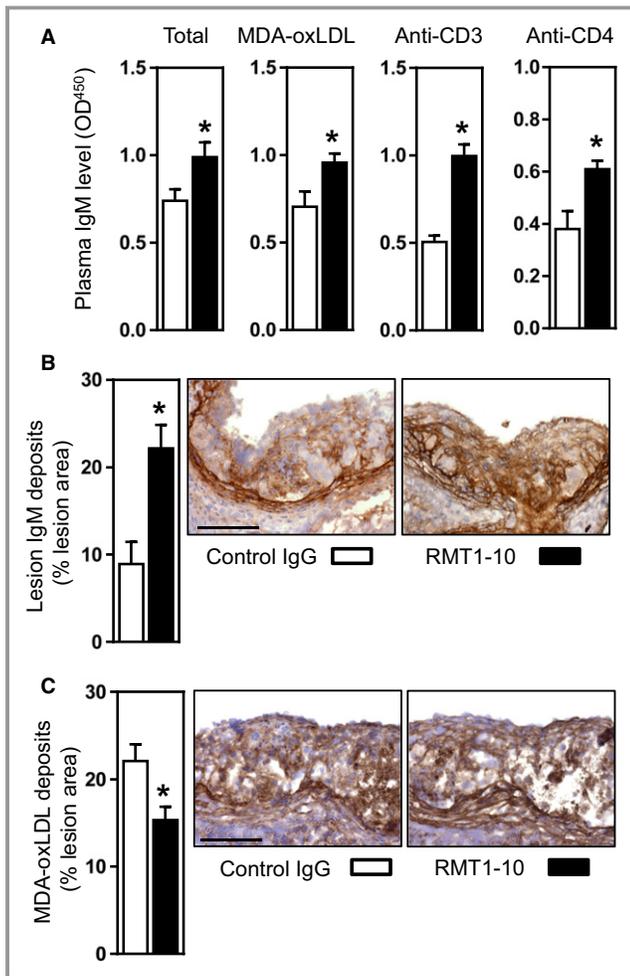


Figure 3. Anti-TIM-1 (RMT1-10) antibody treatment increases plasma IgM levels and lesion IgM deposits. ELISA showed that RMT1-10 treatment (A) increased plasma levels of total, MDA-oxLDL, anti-CD3 and anti-CD4 IgM antibodies. Immunohistochemical analysis showed (B) increased IgM deposits and (C) reduced MDA-oxLDL accumulation in atherosclerotic lesions. Data represent mean±SEM with representative photomicrographs in (B and C). * $P<0.05$, unpaired t test, $n=12$ in control (control IgG-treated) and $n=14$ in test (RMT1-10-treated) groups. IgM indicates immunoglobulin M; MDA, Malondialdehyde; oxLDL, oxidized low density lipoprotein.

Figure 8A), whilst the increase in the spleen did not reach statistical significance ($P>0.05$; Figure 8A). TIM-1⁺ B1a cells represented approximately half of the B1a cell population, and their number increased by nearly 2-fold in the peritoneal cavity and by 55% in the spleen ($P<0.05$; Figure 8B). TIM-1⁺IgM⁺ B1a cells were increased by ≈3-fold in the peritoneal cavity and by 85% in spleen ($P<0.05$; Figure 8C). For TIM-1⁺IL-10⁺ B1a cells, an ≈3-fold increase was observed in the peritoneal cavity ($P<0.05$; Figure 8D), but an increase in spleen did not reach statistical significance ($P>0.05$; Figure 8D). TIM-1⁺IgM⁺IL-10⁺ B1a cells in the peritoneum and

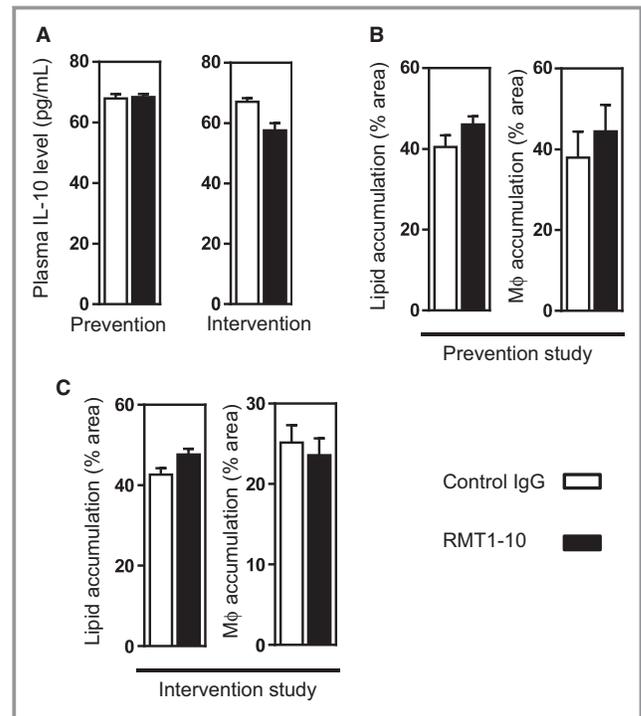


Figure 4. RMT1-10 treatment do not affect plasma IL-10 and lesion percent ratio of lipid and macrophage. ApoE^{-/-} mice were treated with RMT1-10 antibody at the beginning of an 8-week high-fat diet (prevention study). Some ApoE^{-/-} mice were fed a HFD for 6 weeks and treated with RMT1-10 antibody for 8 more weeks (intervention study). A, Plasma IL-10 levels were determined at the completion of experiment. Percent area of lipid and macrophage (Mφ) was unaffected in (B) the prevention study as well as (C) intervention study. Data represent mean±SEM unpaired t test, $n=13$ in control (control IgG-treated) and $n=16$ in test (RMT1-10-treated) groups. IL-10 indicates interleukin-10; IgM, immunoglobulin M.

spleen were significantly increased ($P<0.05$; Figure 8E). The finding that RMT1-10 treatment increased TIM-1⁺ IgM⁺ B1a cells in mice with established lesion is in agreement with our results in the atherosclerosis development experiment. Treatment also attenuated intimal lesion size, by 33% and lipid accumulation by 23% ($P<0.05$; Figure 8F); immunohistochemical comparisons indicated a 34% reduction in lesion macrophages ($P<0.05$; Figure 8G). Similar to the prevention study, the ratio of lipid and macrophage accumulation in the intervention study was also not affected (Figure 4C). IgM deposition in lesions was also increased by 70% by RMT1-10 treatment, and accumulated MDA-oxLDL was reduced by 46% ($P<0.05$; Figures 9A and 9B). Treatment reduced the number of apoptotic cells in lesions by 32% ($P<0.05$) and necrotic cores by 21% ($P<0.05$; Figures 9C and 9D). Lesion MCP-1, VCAM-1, and IL-1β were also reduced, by 59%, 36%, and 35%, respectively, ($P<0.05$; Figures 10A through 10C), as were lesion CD4⁺ and CD8⁺ T cells, by 36% and 49%, respectively, ($P<0.05$; Figures 10D and 10E).

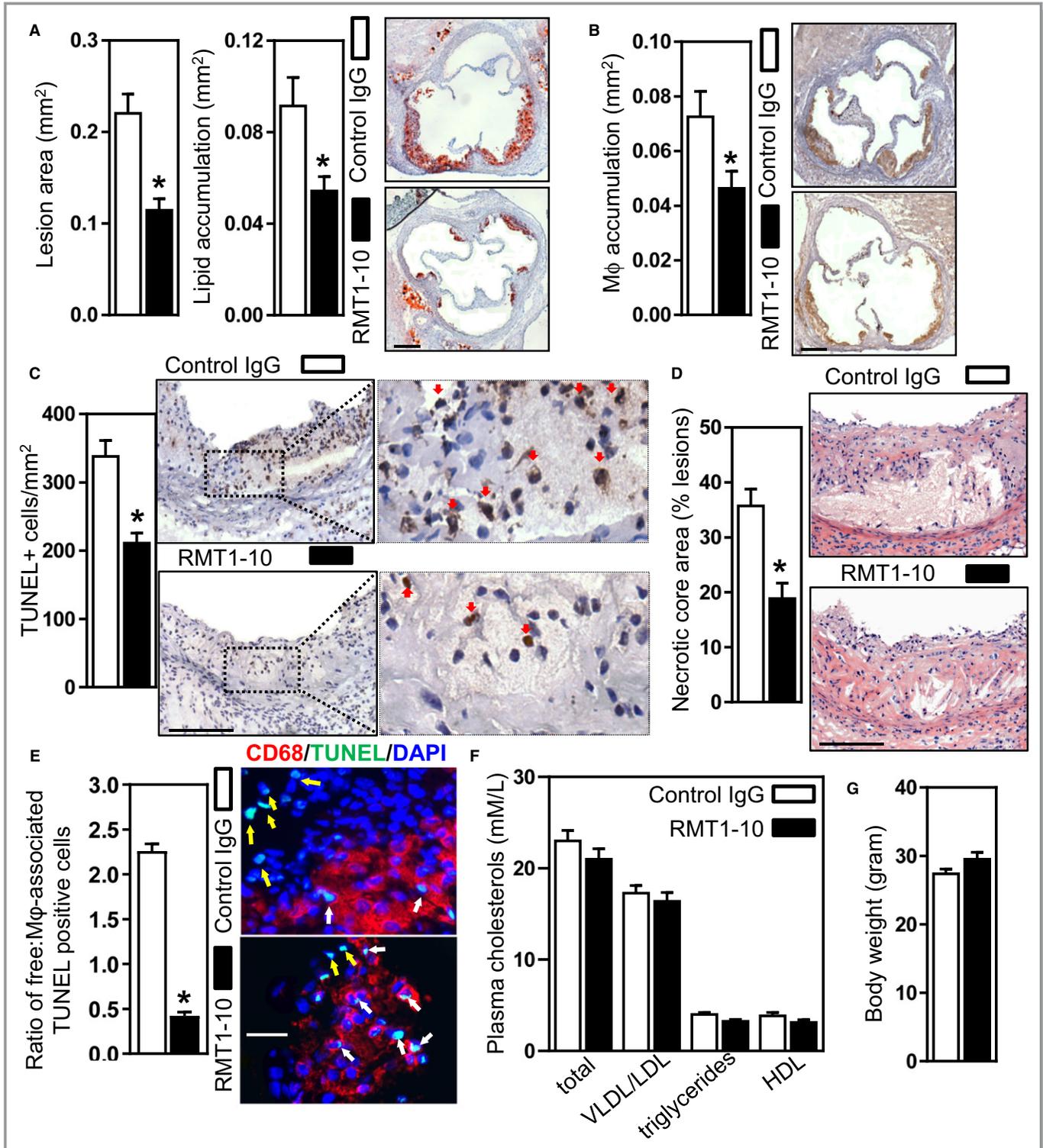


Figure 5. RMT1-10 treatment reduces atherosclerosis development. ApoE^{-/-} mice were treated with RMT1-10 antibody at the beginning of 8 week. Histological staining with Oil Red O showed (A) reduced total intimal lesion area and lesion lipid accumulation and immunohistochemical staining with CD68 showed reduced lesion macrophage (Mφ) accumulation in RMT1-10-treated mice. RMT1-10 treatment reduced (C) apoptotic cells identified as TUNEL-positive cells and (D) necrotic cores identified as H&E stained acellular areas and also improved (E) efferocytosis assessed by free (yellow arrow) and Mφ-associated (white arrow) apoptotic bodies. RMT1-10 treated mice and control IgG treated mice showed comparable (F) plasma cholesterol and (G) body weight. Data represent mean±SEM with representative photomicrographs in (A through D). **P*<0.05, unpaired t test, n=13 in control (control IgG-treated) and n=16 in test (RMT1-10-treated) groups. DAPI indicates 4',6-diamidino-2-phenylindole; IgG, immunoglobulin G; LDL, low density lipoprotein cholesterol; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; vLDL, very low density lipoprotein cholesterol.

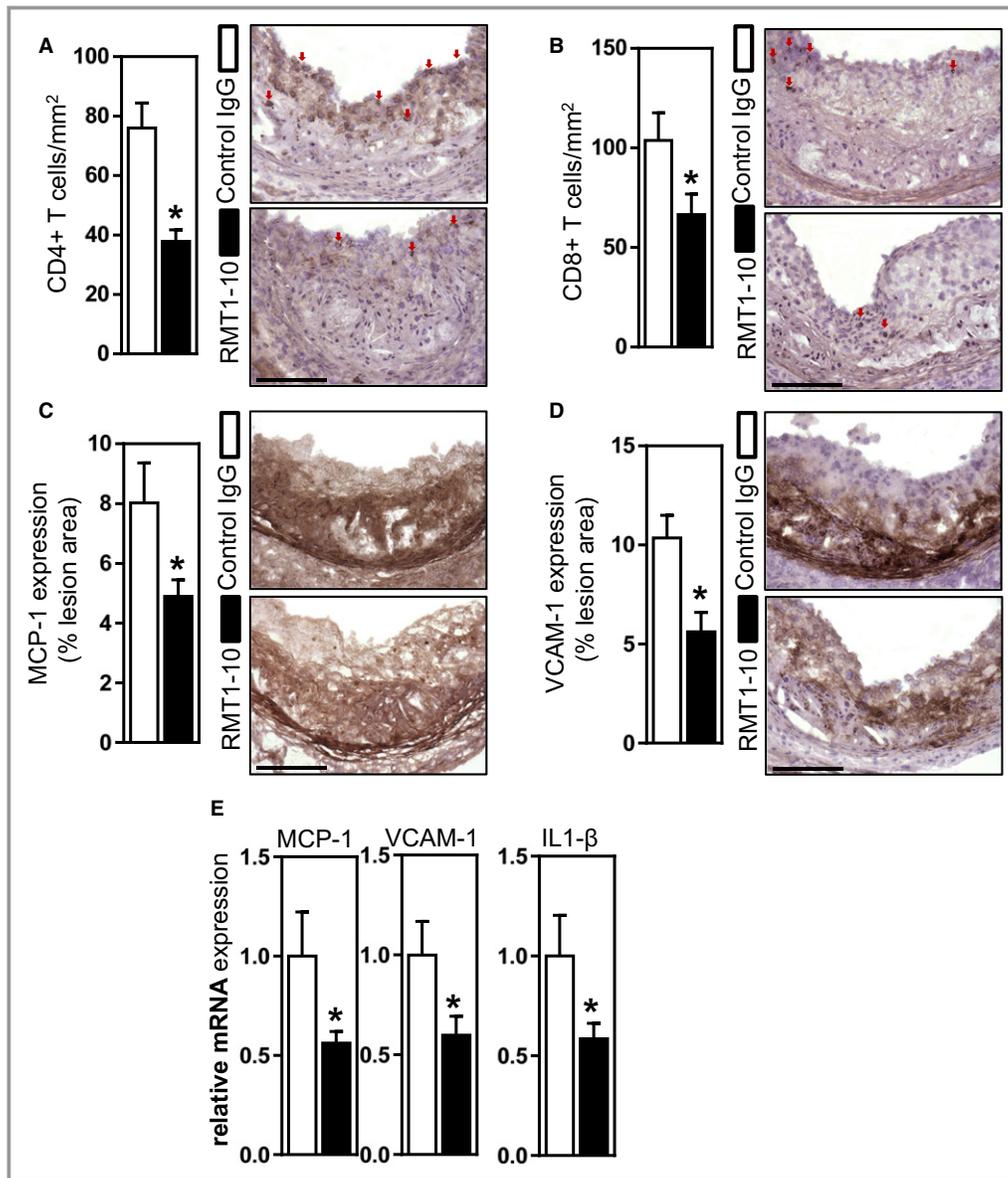


Figure 6. Anti-TIM-1 (RMT1-10) antibody treatment reduces lesion T cell infiltrates and arterial inflammation. Aortic sinus lesions stained with anti-CD4 and anti-CD8 antibodies showed reduced accumulation of (A) CD4⁺ T cells and (B) CD8⁺ T cells in mice treated with RMT1-10 antibody. Immunohistochemical staining showed reduced expression of leukocyte-attractants, (C) monocyte chemoattractant protein-1 (MCP-1) and (D) vascular cell adhesion molecule 1 (VCAM-1) proteins in RMT1-10 treated mice. Real-time PCR analysis using aortic arch mRNA showed (E) reduced mRNA expression of MCP-1, VCAM-1 and IL-1 β in RMT1-10 treated mice. Data represent mean \pm SEM with representative photomicrographs in (A through D). * P <0.05, unpaired t test, n =11 to 14/group. n =11 in control (control IgG-treated) and n =13 in test (RMT1-10-treated) groups. IL-1 β indicates interleukin-1 β ; IgG, immunoglobulin G; MCP-1, Monocyte chemoattractant protein-1; VCAM-1, vascular cell adhesion molecule-1.

Discussion

In this study we demonstrate that B1a cells express TIM-1 and that they may be therapeutically harnessed by their selective in vivo expansion with anti-TIM-1 antibody (RMT1-10) to attenuate atherosclerosis. Treatment of mice with RMT1-10 induced a sustained increase in B1a cells which attenuated not

only atherosclerosis development but also progression of developed atherosclerosis. Our data demonstrate that RMT1-10 therapy may be used to attenuate both development and progression of atherosclerosis by selectively expanding the IgM⁺ and IgM⁺ IL-10⁺ B1a cells. Its effects on lesions in vivo are critically dependent on B1a cells because their depletion by splenectomy abrogated the atheroprotective action of RMT1-10.

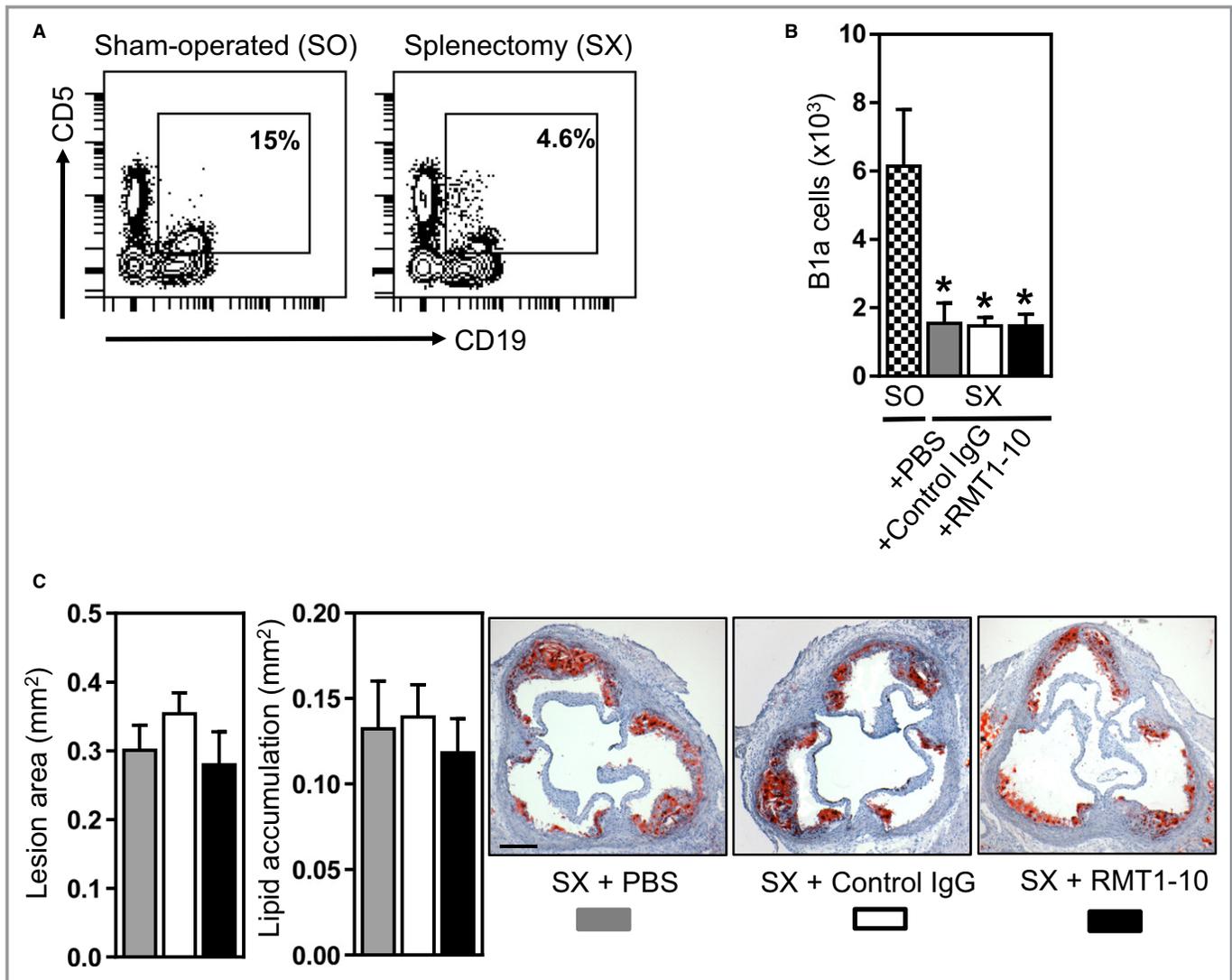


Figure 7. Anti-TIM-1 (RMT1-10) antibody treatment fails to protect against atherosclerosis development in splenectomized mice. A, Representative FACS plots show reduced peritoneal B1a cell in mice with splenectomy. Splenectomized ApoE^{-/-} mice were treated with RMT1-10 during 8 week HFD. FACS analysis showed that (B) reduced peritoneal B1a cells in all splenectomized mice. Atherosclerosis assessment by Oil Red O-staining showed that RMT1-10 treatment did not reduce (C) total intimal lesion area and lipid accumulation in splenectomized mice. Data represent mean±SEM with representative photomicrographs in (C). **P*<0.05 compared with sham-operated (SO) group, one-way ANOVA with Bonferroni post-test, n=5 to 6/group. IgG indicates immunoglobulin G; and RMT1-10, T-cell immunoglobulin mucin domain-1 ligation by anti-TIM-1 monoclonal antibody.

Fetal liver-derived peritoneal B1a cells are a self-renewal type of innate lymphoid cells. In the absence of the spleen generated by genetic or surgical approaches, peritoneal B1a cells are selectively diminished without affecting B1b and B2 cells and serum total and viral-specific IgM levels are reduced.¹⁸ Chimeric mice that received fetal liver cells, not bone marrow cells selectively restored peritoneal B1a cells and increased viral-specific IgM levels, indicating that B1a B cells originating from the fetal liver require the spleen for their survival, maintenance, and effector function, particularly IgM production.¹⁸ We have reported that B1a B cells transferred to splenectomized ApoE-deficient mice decreased splenectomy-

aggravated atherosclerosis by restoring natural IgM antibodies.¹ In the present study, we show that atherosclerosis fails to be ameliorated when splenectomized ApoE-deficient mice were treated with RMT-10 antibody. Collectively, we conclude that RMT-10 antibody treatment reduces atherosclerosis by expansion of natural IgM producing B1a cells.

TIM-1 antibodies can be either an agonist or antagonist antibody that differentially modulate immune responses.¹⁷ Anti-TIM-1 antibody (Clone RMT1-10) ameliorates Experimental autoimmune encephalomyelitis (EAE),¹⁷ allergic asthma,³⁷ glomerulonephritis³⁸ and corneal allograft rejection.³⁹ In contrast another anti-TIM-1 antibody (clone 3B3) enhances

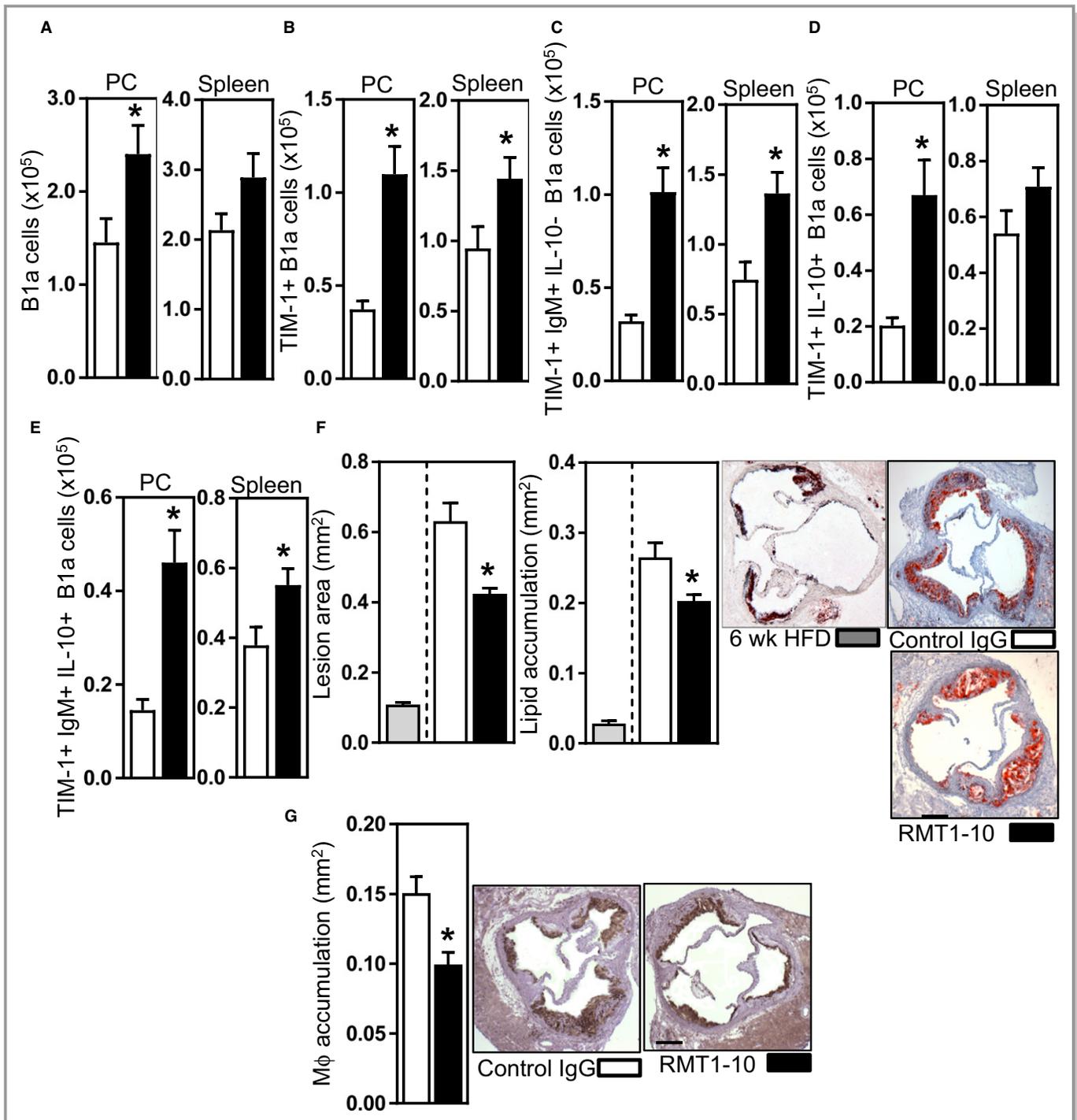


Figure 8. Anti-TIM-1 (RMT1-10) antibody reduces established atherosclerosis. ApoE^{-/-} mice were fed a HFD for 6 weeks to establish atherosclerosis before RMT1-10 treatment for 8 weeks. Fluorescence-activated cell sorting analysis showed increased (A) B1a cells, (B) TIM-1⁺ B1a cells, (C) TIM-1⁺IgM⁺ B1a cells, (D) TIM-1⁺IL10⁺ B1a cells and (E) TIM-1⁺IgM⁺IL10⁺ B1a cells in the peritoneal cavity (PC) and spleen in RMT1-10-treated mice. Atherosclerosis assessment by Oil Red O-staining showed reduced (F) total intimal lesion area and lipid accumulation and immunohistochemical analysis using anti-CD68 antibody showed reduced (G) macrophage (Mφ) accumulation in RMT1-10 treated mice. **P*<0.05 compared with IgG control, unpaired t test. Data represent mean±SEM with representative photomicrographs in (F and G). **P*<0.05, unpaired t test, n=11 in untreated (6 weeks HFD), n=13 in control (control IgG-treated) and n=15 in test (RMT1-10-treated) groups. HFD indicates high fat diet; IL-10, interleukin-10; IgG, immunoglobulin G; IgM, immunoglobulin M; PC, peritoneal cavity; TIM-1, T-cell immunoglobulin and mucin domain-1.

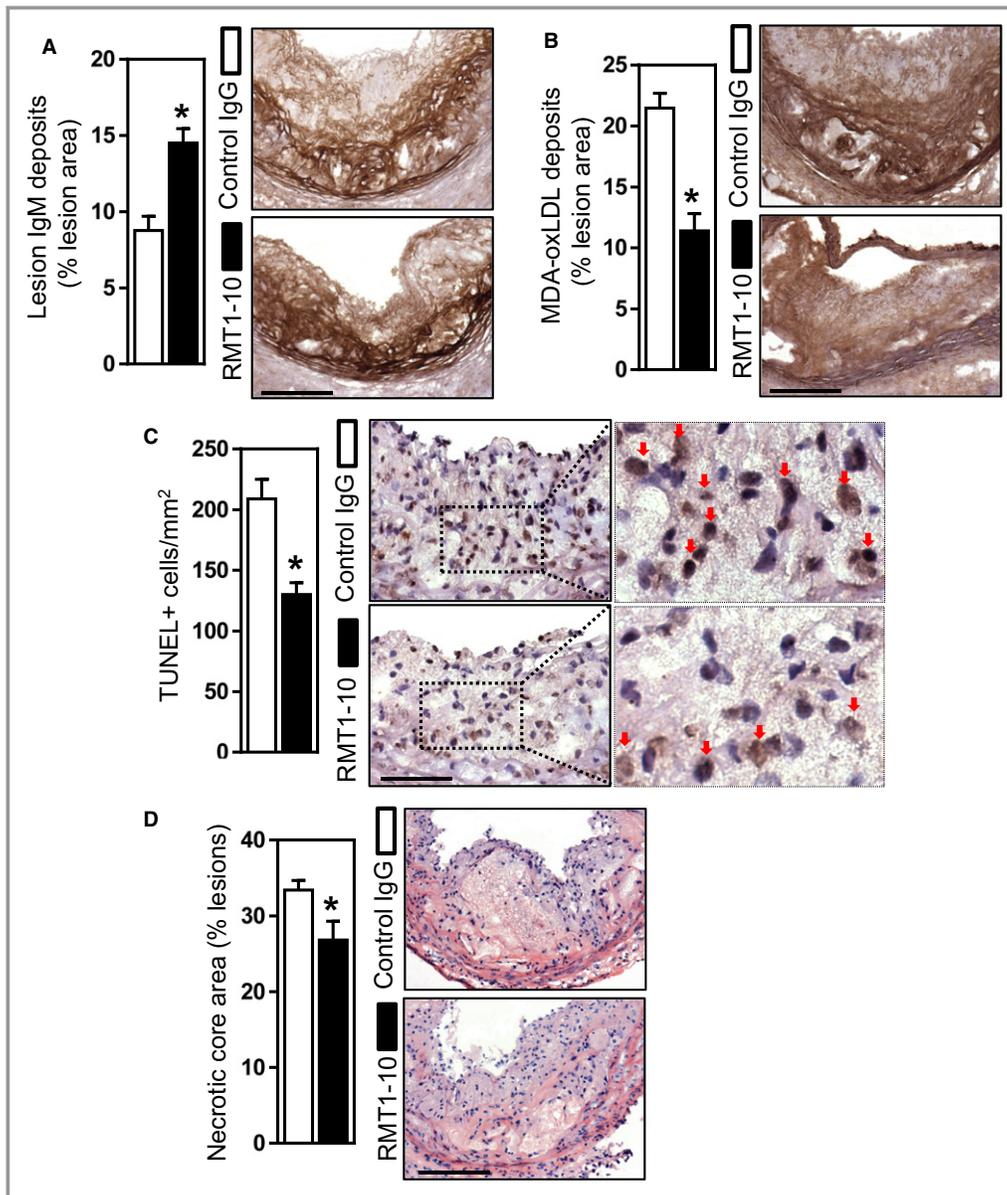


Figure 9. Anti-TIM-1 (RMT1-10) antibody treatment reduces lesion necrosis and apoptosis in ApoE^{-/-} mice with established atherosclerosis. HFD-fed mice were treated with RMT1-10 antibody. Immunohistochemical analysis of aortic sinus atherosclerotic lesions showed (A) increased IgM deposits and (B) reduced oxidized-LDL (oxLDL) accumulation in RMT1-10-treated mice. Apoptotic cells as identified by (C) TUNEL-positive cells and necrotic cores as identified by (D) acellular areas in H&E staining were reduced in RMT1-10-treated mice. Data represent mean±SEM with representative photomicrographs. **P*<0.05, unpaired t test, n=11 in control (control IgG-treated) and n=13 in test (RMT1-10-treated) groups. IgG indicates immunoglobulin G; IgM, immunoglobulin M; MDA, Malondialdehyde; oxLDL, oxidized LDL; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling.

T cell proliferation and Th2 effector function.^{17,40,41} HFD-fed LDLR-deficient mice treated with anti-TIM-1 antibody (clone 3D10) accelerated atherosclerosis by ≈50% via increased aortic CD4 T cells.⁴² In our present study, we have shown that RMT1-10 ameliorated atherosclerosis by expansion of IgM-secreting B1a cells. Therefore, it is necessary to select the appropriate TIM-1 antibody for atherosuppression.

RMT1-10 treatment seems to induce atheroprotective B1a B cells as well as immune-modulating regulatory B cells,¹⁴ however IL-10+ regulatory B cells do not contribute to atherosclerosis⁴³ despite the finding that B10 cells are potent immunosuppressive cells in colitis⁴⁴ and other autoimmune inflammatory disorders.^{45,46} The majority of IL-10 is produced by macrophages and dendritic cells and

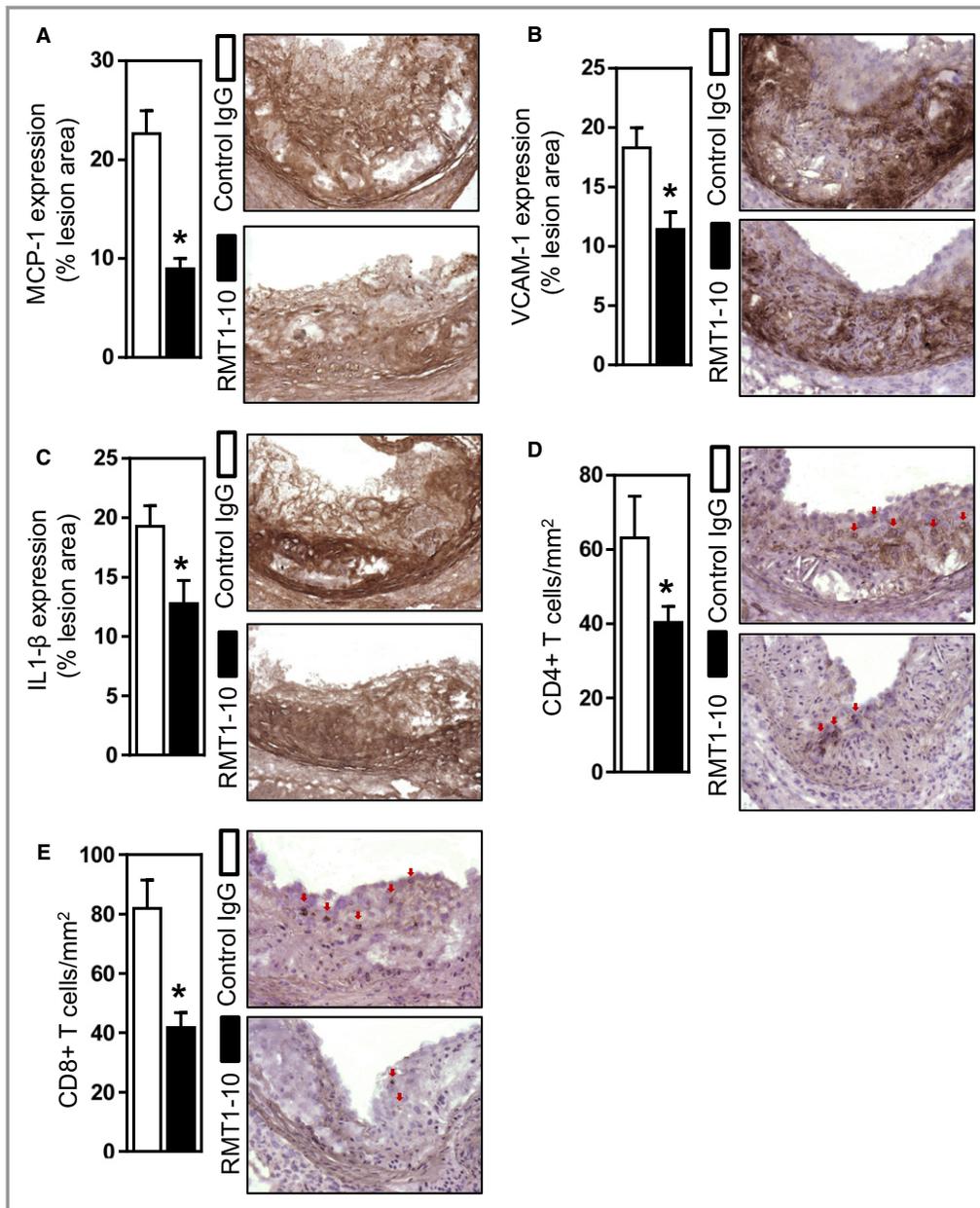


Figure 10. Anti-TIM-1 (RMT1-10) antibody treatment reduces lesion T cell infiltrates in ApoE^{-/-} mice with established atherosclerosis. Immunohistochemical analysis showed that expression of leukocyte attractants, (A) monocyte chemoattractant protein-1 (MCP-1) and (B) vascular cell adhesion molecule 1 (VCAM-1) and inflammatory marker, (C) IL-1-β was reduced and accumulation of (D) CD4+ and (E) CD8+ T cells in lesions was also decreased in RMT1-10-treated mice. Data represent mean±SEM with representative photomicrographs. **P*<0.05, unpaired t test, n=13 in control (control IgG-treated) and n=15 in test (RMT1-10-treated) groups.

with lessor amounts produced by T and B cells⁴⁷ including B1a cells.^{5,8} Consistent with these reports, we found that RMT1-10 treatment increases IL-10-expressing B1a cells without affecting plasma IL-10 levels. B1a-derived IL-10 may regulate B1a-derived IgM production as suggested by the report that B cell-derived IL-10 promotes paracrine as well as autocrine IgM production.⁴⁸

Unlike global depletion of the total B cell population or the B2 cell population to attenuate atherosclerosis, we focused on expanding atheroprotective B1a cells by targeting TIM-1, to minimize perturbation of the immune system. TIM-1 is most abundantly expressed on B cells and TIM-1 ligation with RMT1-10 markedly expands spleen IL-10-expressing regulatory B cells.¹⁴ As a substantial number of these B cells that

express CD1d and CD5 markers and regulatory B cells also express TIM-1,¹⁴ we initially examined CD1d expression on TIM-1+ IgM+ IL-10+ B1a cells and CD1d+ CD5+ regulatory B cells in spleen and peritoneal cavities as CD1d^{hi} CD5⁺ regulatory B cells has also recently been found to be highly represented in peritoneal B1a cells.⁴⁴ Our data showed that TIM-1+ IgM+ IL-10+ B1a cells in the peritoneal cavity and spleen were also found to express CD1d and are most likely similar, to the recently described peritoneal and spleen B10 cells.^{5,44} Chronic RMT1-10 treatment expands B1a cells in the spleen and peritoneal cavity, greatly increases plasma IgM levels and accumulation of IgM in developing and developed atherosclerotic lesions whilst decreasing accumulation of oxidized LDL. IgM natural antibodies binding oxLDL protect against experimental atherosclerosis^{1,49} and levels are inversely related to carotid artery atherosclerosis in humans.⁵⁰ RMT1-10 treatment appears to specifically target TIM-1⁺ B1a cells, as no changes were observed in other lymphocyte populations, including B2 cells; the latter is consistent with the treatment not affecting plasma IgG or anti-MDA-LDL IgG levels.

In addition to effective removal of ox-LDL by natural IgM antibodies, effective efferocytosis also facilitates clearance of apoptotic cells in the lesions. TIM-1 expressed on phagocytes is involved in the recognition of apoptotic cells and initiation of efferocytosis by interacting with phosphatidylserine expressing apoptotic cells.⁵¹ Mice expressing defective TIM-1 fail to phagocytose apoptotic cells in acute renal injury consistent with the critical role of TIM-1 in efferocytosis.⁵² Unlike 3B3 and 3D10 agonist antibody clones, RMT1-10 does not block binding of phosphatidylserine-expressing apoptotic cells to TIM-1-expressing phagocytes⁵³ indicating a potential role of RMT1-10 in facilitating efferocytosis. It is supported by our results where RMT1-10 treatment improves lesion efferocytosis and with the results of Foks et al where 3D3 treated mice showed increased apoptosis and impaired efferocytosis in 3B3 treated mice.⁴²

The sustained selective expansion of TIM-1+ IgM+ and TIM-1+ IgM+ IL-10+ B1a cells in the peritoneal cavity and spleen by RMT1-10 suppressed atherosclerosis and reduced apoptotic cell numbers in lesions and necrotic core size; effects consistent with the increases in plasma and lesion IgM. Atherosclerotic mice not capable of producing IgM have greater numbers of apoptotic cells within atherosclerotic lesions.^{1,49} These observations are consistent with a scavenger function for natural IgM produced by B1a cells in mopping up apoptotic debris.⁵⁴ Antigen-activated T cells are known to aggravate atherosclerosis.⁵⁵ By binding ox-LDL, IgM can also modify the antigen environment, reducing the availability of such antigens to antigen-presenting cells for processing and presentation to T cells.⁵⁴

Reduced CD4⁺ and CD8⁺ T cells in atherosclerotic lesions suggest that increased anti-CD3 and CD4 IgM polyclonal

antibodies may have contributed to a reduction of these atherogenic T cells^{28,56} in atherosclerotic lesions. Anti-CD3 monoclonal antibody is immunosuppressive, and its therapeutic applications suggested in autoimmune diseases, tissue rejections and T cell carcinomas. Both development and progression of diabetes mellitus are effectively prevented by anti-CD3 antibody^{57,58} and teplizumab (anti-CD3 antibody) improved patients with Type I diabetes mellitus resulting from auto-destruction of beta cells by T cells.⁵⁹ We have shown in this study that RMT1-10 treatment not only expands B1a cells but also increases plasma levels of total and polyclonal IgM antibodies (oxLDL, anti-CD3 and anti-CD4).

In addition to suppressive effects of RMT1-10 treatment on T cells, macrophage accumulation in lesions was also reduced. This is associated with similar reductions in lesion MCP-1 and VCAM-1, which may contribute to the reduction in macrophage accumulation. MCP-1 is produced by vascular smooth muscle cells and macrophages, is chemotactic for monocytes/macrophages, and preventing its expression attenuates atherosclerosis development.⁶⁰ Similarly, VCAM-1 contributes to macrophage accumulation in developing lesions.⁶¹ The reduction in MCP-1 and VCAM-1 expression is most likely the consequence of reduced T cell and macrophage activation; macrophage and T cell-derived cytokines are reduced by RMT1-10 treatment and tumor necrosis factor α , IL-6 and IL-1 β are potent inducers of MCP-1 expression,⁶²⁻⁶⁴ whilst tumor necrosis factor α and IL-1 β are also known to induce VCAM-1 expression.^{65,66}

We showed that antagonist antibody-mediated in-vivo expansion of B1a cells retards both development and progression of atherosclerosis, but there are limitations in the current study. Firstly, effects of sex hormones have been well documented in immune responses⁶⁷ and atherosclerosis development.⁶⁸ Further studies using female mice are warranted to confirm similar effect in the presence of oestrogen. Secondly, HFD-feeding on genetically modified atherogenic mice generates different atherosclerosis via site-specific and time-dependent manners.⁶⁹ Thus, extensive studies are necessary to determine effect of RMT1-10 treatment on atherosclerosis at different anatomical location and at different time points in pre-clinical studies.

Conclusion

In summary, we have demonstrated that targeting TIM-1 on B1a cells inhibits atherosclerosis development and progression by inducing expansion of TIM-1⁺ IgM⁺ B1a cell population and increasing levels of polyclonal IgM, an important mechanisms of atherosclerosis suppression. Although the mechanism by which ligation of TIM-1 on B1a cells leads to their expansion remains to be clarified, our data indicate that

expansion of B1a cells can be used as a promising therapeutic target to attenuate development and progression of atherosclerosis-related vascular disorders.

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Disclosures

None.

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