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Hidde Douna [a], Virginia Smit [a], Gijs H.M. van Puijvelde [a], Mate G. Kiss [b,c], Christoph J. Binder [b,c], Ilze Bot [a], Vijay K. Kuchroo [d], Andrew H. Lichtman [e], Johan Kuiper [a], Amanda C. Foks [a,*]

[a] Division of BioTherapeutics, LACDR, Leiden University, Einsteinweg 55, 2333CC, Leiden, the Netherlands
[b] Department of Laboratory Medicine, Medical University of Vienna, Vienna, 1090, Austria
[c] CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria
[d] Department of Neurology, Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, 02115, USA
[e] Department of Pathology, Brigham and Women's Hospital, Boston, MA, 02115, USA

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ABSTRACT

Background and aims: Increasing evidence has shown that immune checkpoint molecules of the T-cell immunoglobulin and mucin domain (Tim) family are associated with diverse physiologic and pathologic processes. Previous studies of the role of Tim-1 in atherosclerosis using anti-Tim-1 antibodies have yielded contradictory results. We thus aimed to investigate atherosclerosis development in *Tim-1* deficient mice.

Methods: Mice with a specific loss of the Tim-1 mucin-domain (*Tim-1*$^{\Delta mucin}$) and C57BL/6 (WT) mice received a single injection of a recombinant adeno-associated virus encoding murine Pcsk9 (rAAV2/8-D377Y-mPcsk9) and were fed a Western type diet for 13 weeks to introduce atherosclerosis.

Results: *Tim-1*$^{\Delta mucin}$ mice developed significantly larger lesions in the aortic root compared to WT mice, with significantly more macrophages and a trend towards a larger necrotic core. Furthermore, *Tim-1*$^{\Delta mucin}$ mice showed a significant loss of IL-10^{+} B cells and regulatory B cell subsets and increased pro-atherogenic splenic follicular B cells compared to WT mice. Moreover, *Tim-1*$^{\Delta mucin}$ mice displayed a dramatic reduction in Th2-associated immune response compared to controls but we did not observe any changes in humoral immunity.

Conclusions: In summary, *Tim-1*$^{\Delta mucin}$ mice displayed a profound impairment in IL-10^{+} B cells and an imbalance in the Th1/Th2 ratio, which associated with exacerbated atherosclerosis.

1. Introduction

Regulation of immune responses represents a promising option for the treatment of atherosclerosis [1]. Activation of the immune system is tightly controlled by immune checkpoint proteins and these molecules comprise an interesting group of therapeutic targets [2]. Immune checkpoint proteins are comprised of a diverse family of molecules, each with its own target cells and pathways. In general, immune checkpoint proteins are divided into stimulatory or inhibitory coreceptors, which are often associated with a proatherogenic and atheroprotective role, respectively [2]. Earlier data show that immune checkpoint molecules of the T-cell immunoglobulin and mucin domain (TIM for humans, Tim for mice) family are associated with a number of disorders including asthma, allergy and autoimmunity [3]. Eight murine members of the *Tim* gene family have been identified (*Tim-1-8*), while in humans only three orthologues have been characterized (*TIM-1, -3, and -4*). Structurally, Tim molecules are composed of an immunoglobulin V domain, a mucin-like domain, a transmembrane domain and a cytoplasmic tail [3]. There are distinct ligands for each Tim protein, however, there is some overlap between Tim-1 and Tim-4 since both molecules are able to bind phosphatidylserine which is expressed by apoptotic cells [4]. Additionally, it has been shown that Tim-4 is able to interact with Tim-1 as a ligand [5]. The cellular expression pattern of Tim proteins is quite broad, including T cells, B cells, macrophages and dendritic cells (DCs), highlighting the central role Tim molecules can play in the immune response.

We have previously explored the effects of Tim-1, Tim-3 and Tim-4 in atherosclerosis and identified that antibody-mediated blockade of any of these Tim molecules results in exacerbated atherosclerosis, indicating a general protective role of Tim-signaling in atherosclerosis [6,7]. In contrast, others have recently shown that by using a different antibody clone, Tim-1 antagonism resulted in attenuated atherosclerosis [8]. Tim-1 is an immune checkpoint protein which is primarily expressed on CD4^{+} helper type 2 cells [9], dendritic cells and regulatory B cells [10]. Principal studies suggested that Tim-1 is a stimulatory coreceptor since

* Corresponding author. Division of BioTherapeutics, LACDR, Leiden University, Einsteinweg 55, 2333CC, Leiden, the Netherlands.
E-mail address: a.c.foks@lacdr.leidenuniv.nl (A.C. Foks).

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it induced T cell proliferation and cytokine production [9]. Later studies, however, showed that Tim-1 has a more complex immune checkpoint function [5,11]. It has been demonstrated that the administration of low doses of a soluble Tim-1 ligand (Tim-4) results in significant T cell inhibition, while higher doses increase T cell proliferation [5]. Additionally, it was shown that differential engagement by two Tim-1 antibodies could result in T cell expansion or inhibition [11]. These data clearly demonstrate that Tim-1 signaling is highly dependent on ligand density and the extent of Tim-1 engagement. This could potentially explain the differential effects of antagonistic Tim-1 antibodies on atherosclerosis [6,8]. Indeed, the anti-Tim-1 antibody used by Hosseini et al. (RMT1-10) has a low binding avidity and might be partial agonistic [11], while the antibody used in our previous experiments (3D10) is described as a non-activating antibody [12].

The contradictory results obscure the precise contribution of Tim-1 during atherosclerosis development and warrant additional research. To circumvent the complexities associated with antibody binding avidity and functionality, we aimed to examine the effects of Tim-1 in atherosclerosis using Tim-1-deficient mice. Earlier work showed that complete Tim-1 deficiency fails to show any marked phenotype [13,14]. However, others have previously shown that the mucin domain of Tim-1 has a crucial biological function in Tim-1 signalling [15]. Mice with a specific loss of the Tim-1 mucin-domain (*Tim-1*$^{\Delta mucin}$) retain the ligand binding IgV structure and express Tim-1 at normal levels. These mice do demonstrate a profound defect in regulatory B cells with associated development of systemic autoimmunity [15]. In this study, we thus examined the effect of defective Tim-1 signaling in *Tim-1*$^{\Delta mucin}$ mice on atherosclerosis.

2. Materials and methods

2.1. Animals

Female C57BL/6 (WT) and Tim-1 mucin domain-deficient (*Tim-1*$^{\Delta mucin}$) mice [15] were bred in house and were kept under standard laboratory conditions. *Tim-1*$^{\Delta mucin}$ mice were provided by Dr. V. Kuchroo (Brigham and Women's Hospital). Mice were fed a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Diet and water were provided *ad libitum*. During the experiments, mice were weighed, and blood samples were obtained by tail vein bleeding. At the end of the experiments, mice were anaesthetized by a subcutaneous injection of a cocktail containing ketamine (40 mg/mL), atropine (50 μg/mL), and sedazine (6.25 mg/mL). Mice were exsanguinated by femoral artery transection followed by perfusion with PBS through the left cardiac ventricle. All animal work was approved by the Leiden University Animal Ethics Committee and the animal experiments conformed with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

2.2. Pcsk9-induced atherosclerosis

To induce atherosclerosis in WT and *Tim-1*$^{\Delta mucin}$ mice, 8–9 week old female mice were administered a single i.v. injection of rAAV2/8-D377Y-m*Pcsk9* (5×10 [11] genome copies/mouse) [16]. This results in the rapid overexpression of proprotein convertase subtilisin/kexin type 9 (Pcsk9) and a significant decrease in hepatic low-density lipoprotein receptor (Ldlr) [16]. For atherosclerosis development, mice were subsequently fed a WTD for 13 weeks before mice were sacrificed and relevant organs were harvested for analysis.

2.3. Flow cytometry

To obtain single cell suspensions, spleens were mashed through 70 μm cell strainers (Greiner Bio-One), after which erythrocytes were removed with ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA; pH 7.3). Similarly, erythrocytes were lysed from blood samples with ACK lysis buffer. For the analysis of IL-10$^+$ B cells, single cell suspensions were stimulated for 5 h with LPS (50 μg/ml), PMA (50 ng/ml), ionomycin (500 ng/ml) and monensin (2 μM). Secretion of cytokines by T cells was induced with stimulation of PMA, ionomycin and brefeldin A (5 μg/ml). For flow cytometry analysis, Fc receptors of single cell suspensions were blocked with an unconjugated antibody against CD16/CD32. Samples were then stained with a fixable viability marker (ThermoScientific) to select live cells and with anti-mouse fluorochrome-conjugated antibodies (Supplementary Table 1). FACS analysis was performed on a FACSCanto II (Becton Dickinson) and the acquired data were analyzed using FlowJo software.

2.4. Proliferation assay

To measure T cell proliferation, single cell suspensions of splenocytes were obtained by mashing spleens through a 70 μm cell strainers (Greiner Bio-One), after which erythrocytes were removed with ACK lysis buffer. 2×10 [5] splenocytes were cultured in RPMI medium (supplemented with 10% FCS, L-glutamine and streptomycin/penicillin) for 72 h. Cells were cultured with medium, oxidized low-density lipoprotein (oxLDL, 5 μg/ml) or stimulated with anti-CD3 (1.25 μg/ml) and anti-CD28 (1.25 μg/ml). Supernatant was harvested for cytokine analysis before splenocytes were pulsed with 3H-thymidine (0.5 μCi/well, Perkin Elmer, The Netherlands) for the last 16 h. The amount of 3H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). Responses are expressed as the disintegrations per minute (DPM).

2.5. Cytokine determination

Cytokine levels in culture supernatants were measured using a Luminex bead-based multiplex assay (ProcartaPlex, Thermo Fisher Scientific) on a Luminex Instrument (MAGPIX). Recombinant cytokine standards (Thermo Fisher Scientific) were used to calculate cytokine concentrations and data were analyzed using Bio-Rad software.

2.6. Blood and serum analyses

During the experiment, blood was collected from the tail vein. After euthanasia, orbital blood was collected in EDTA-coated tubes. Whole blood cell counts were analyzed using the XT-2000iV hematology analyzer (Sysmex Europe GMBH, Norderstedt, Germany). Serum was acquired by centrifugation and stored at −20 °C until further use. The total cholesterol levels in serum were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). Precipath (Roche/Hitachi) was used as an internal standard. Total serum titers of IgM, IgG1, IgG2c and oxidized LDL-specific antibodies were quantified by ELISA as previously described [17]. Serum levels of Pcsk9 were measured by ELISA according to manufactures protocol (R&D systems).

2.7. Histology

To determine lesion size, cryosections (10 μm) of the aortic root were stained with Oil-Red-O and hematoxylin (Sigma-Aldrich). Sections with the largest lesion plus four flanking sections were analyzed for lesion size. Collagen content in the lesion was assessed with a Masson's trichrome staining according to the manufacturers protocol (Sigma-Aldrich). Corresponding sections on separate slides were also stained for monocyte/macrophage content using a monoclonal rat IgG2b antibody (MOMA-2, 1:1000, AbD Serotec) followed by a goat anti-rat IgG-horseradish peroxidase antibody (1:100, Sigma-Aldrich) and color development. CD4$^+$ and CD8$^+$ T cells were stained using CD4 (RM4-5, 1:90, ThermoFisher) and CD8a (Ly-2, 1:100, eBioscience) antibodies, and a secondary rabbit anti-rat IgG antibody (BA-4001, Vector),

followed by the Vectastain ABC kit (PK-4000, Vector). Color development was achieved using novaRED peroxidase (Vector laboratories) as enzyme substrate. T cells were scored manually. All slides were analyzed with a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems).

2.8. Statistics

All data are expressed as mean ± SEM. Data were tested for significance using a Student's t-test if equal variances and normality could be assumed, otherwise a non-parametric Mann-Whitney test was used. Correlations were determined using Pearson correlation coefficients and significance was determined using linear regression analysis. Probability values of $p < 0.05$ were considered significant. All statistical analysis was performed using GraphPad Prism 7.0.

3. Results

3.1. *Tim-1*$^{\Delta mucin}$ mice display exacerbated atherosclerosis

Since we aimed to investigate the effects of impaired Tim-1 signaling in the context of atherosclerosis, we administered *Tim-1*$^{\Delta mucin}$ mice a single injection of a recombinant adeno-associated virus encoding a gain-of-function for Pcsk9 (rAAV2/8-D377Y-mPcsk9) (Fig. 1A) [16]. This resulted in the rapid induction of circulating Pcsk9 (Supplementary Fig. 1A) and cholesterol (Supplementary Fig. 1B) levels after mice were fed a WTD. During the experiment mice of both groups gained weight. Despite a significant lower body weight at the time of sacrifice compared to WT mice (Supplementary Fig. 1C), we found that *Tim-1*$^{\Delta mucin}$ mice showed a considerable 30% increase in lesion development compared to WT mice (Fig. 1B). We further characterized lesion composition and noticed that the macrophage content was significantly increased in lesions from *Tim-1*$^{\Delta mucin}$ mice compared to WT mice (Fig. 1C). Additionally, there were no differences in collagen content, but we found a trend towards a larger necrotic core area in lesions of *Tim-1*$^{\Delta mucin}$ mice compared to WT mice (Fig. 1D). Notably, relative macrophage, collagen and necrotic core content was not affected. Although we observed reduced CD8$^+$ T cells in the aortic root of *Tim-1*$^{\Delta mucin}$ mice, CD4$^+$ T cells did not differ between the groups and overall only very few T cells were found. Altogether, these data indicate that *Tim-1*$^{\Delta mucin}$ mice developed exacerbated atherosclerosis.

3.2. *Tim-1*$^{\Delta mucin}$ mice show impaired IL-10 production by Bregs

In a previous study, it was demonstrated that the major impairment in *Tim-1*$^{\Delta mucin}$ mice is defective IL-10 production by B cells [15]. To assess if reduced IL-10-producing B cells (Bregs) could have contributed to the increased lesion development in our experiment, we measured circulating IL-10-producing B cells during the experiment. As shown in Fig. 2A, IL-10$^+$ B cells were reduced in *Tim-1*$^{\Delta mucin}$ mice compared to WT mice which reached significance at week 3 and 8. Additionally, the area under the curve showed that the total presence of circulating Bregs during the experiment was significantly reduced in *Tim-1*$^{\Delta mucin}$ mice compared to WT mice (Fig. 2B). Importantly, we show that circulating IL-10$^+$ B cells show an inverse correlation with lesion size and macrophage content (Fig. 2C). And in addition to reduced percentages of IL-10$^+$ B cells, circulating B cells of *Tim-1*$^{\Delta mucin}$ mice also showed lower IL-10 expression per cell (Supplementary Fig. 2A). Subsequently, we found a significant reduction in the percentage of Breg cells in spleens of *Tim-1*$^{\Delta mucin}$ mice at sacrifice (Fig. 2D). A similar trend was observed for absolute numbers of Bregs and IL-10 expression per cell (Supplementary Figs. 2A–B). Furthermore, we determined the level of well-known Breg subsets CD1d^{hi}CD5$^+$ B cells [18] and CD9$^+$ B cells [19,20] and found that these subsets were significantly lower in *Tim-1*$^{\Delta mucin}$ mice when

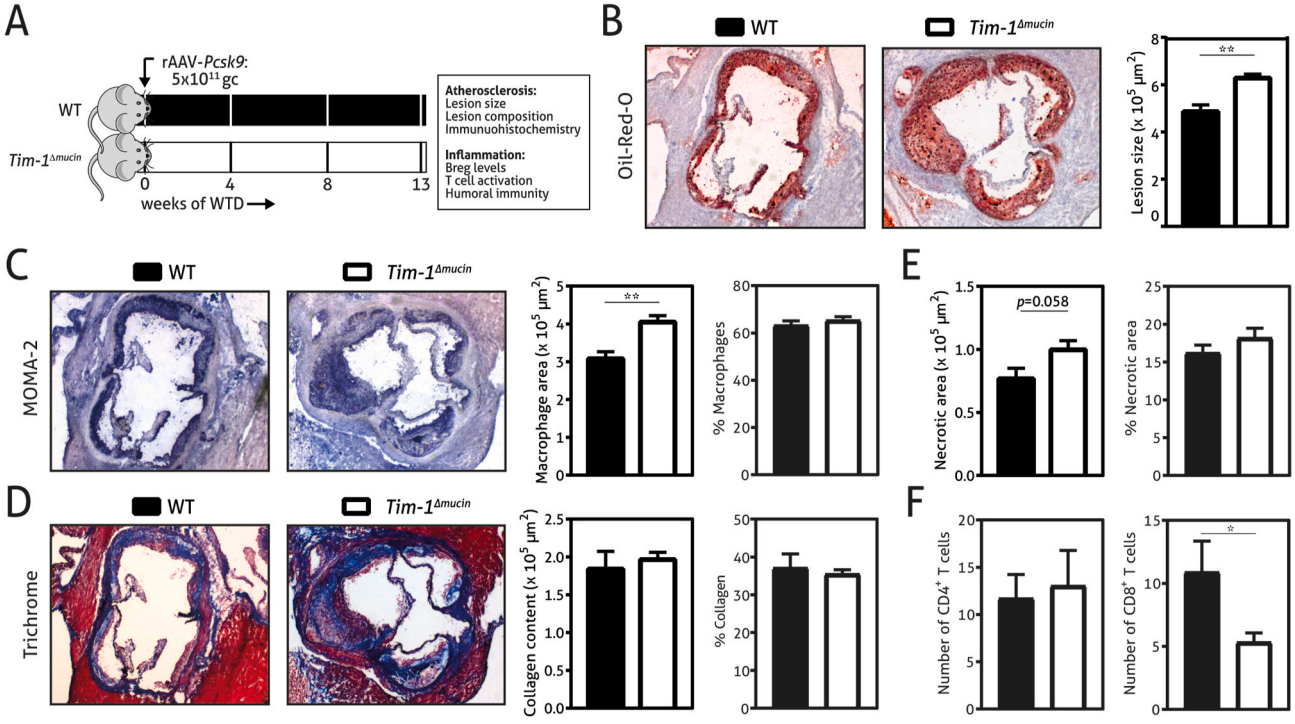

Fig. 1. *Tim-1*$^{\Delta mucin}$ mice display exacerbated atherosclerosis.
(A) C57BL/6 (WT) and Tim-1-mucin domain-deficient (*Tim-1*$^{\Delta mucin}$) mice were administered a single i.v. injection of rAAV2/8-D377Y-m*Pcsk9* and subsequently fed a Western type diet for 13 weeks to induce atherosclerosis. After 13 weeks the aortic root was analyzed for (B) lesion size, which was determined with an Oil-Red-O and hematoxylin staining, (C) macrophage/monocyte content, which was determined with a MOMA-2 antibody and (D) collagen content and (E) necrotic area, which were assessed using a Trichrome staining. (F) CD4$^+$ and CD8$^+$ T cells in the aortic root were manually analyzed. Representative pictures are shown. Data are shown as mean ± SEM (*$p < 0.05$, **$p < 0.01$), n = 12–19/group for A-E, n = 5–8/group for F.

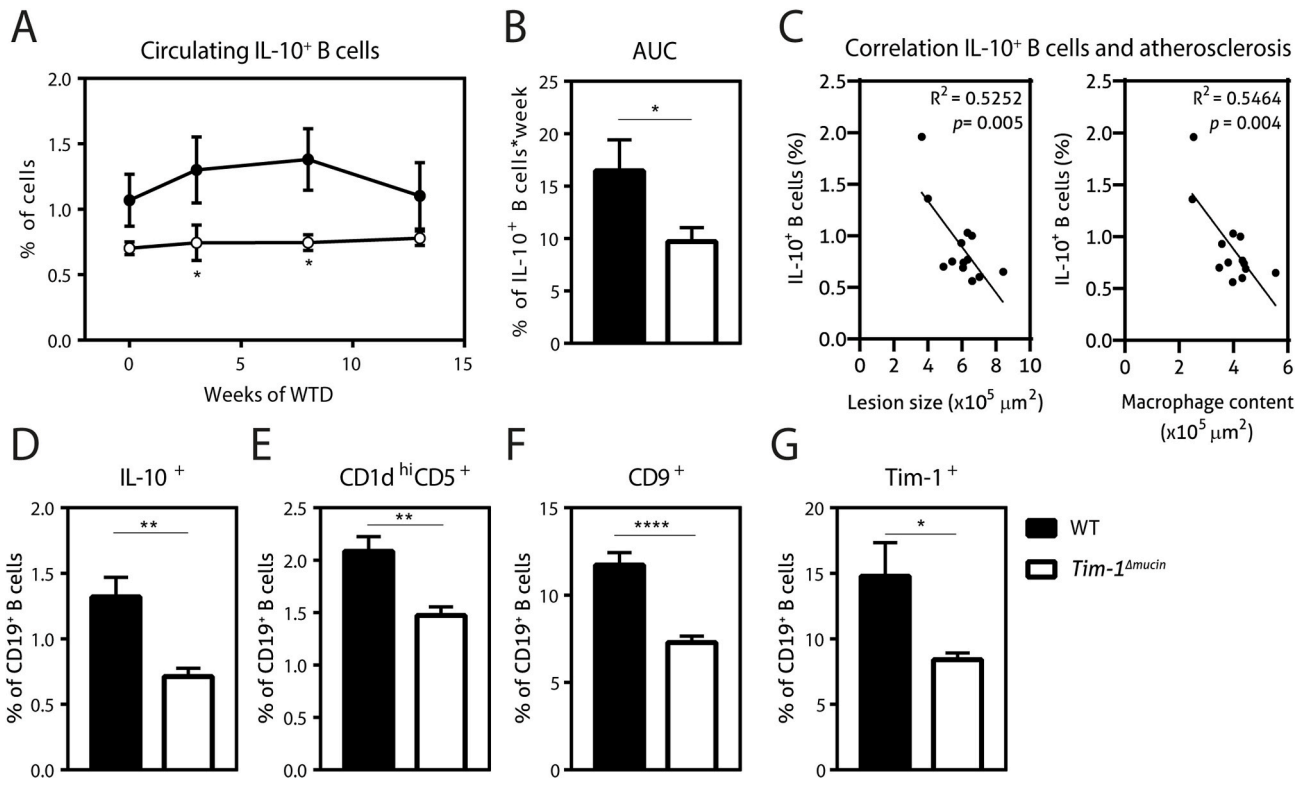

Fig. 2. *Tim-1*$^{\Delta mucin}$ mice show impaired IL-10 production by Bregs.
C57BL/6 (WT) and Tim-1-mucin domain-deficient (*Tim-1*$^{\Delta mucin}$) mice were administered a single i.v. injection of rAAV2/8-D377Y-m*Pcsk9* and subsequently fed a Western type diet for 13 weeks to induce atherosclerosis. (A) During the experiment, blood samples were obtained and single cell suspensions of blood leukocytes were stimulated for 5 h with LPS, PMA, ionomycin and monensin after which the IL-10 positive B cells were determined with flow cytometry. (B) Quantification of the area-under-the curve of circulating IL-10$^+$ B cells during the experiment. (C) Correlation between the percentage of circulating IL-10$^+$ B cells at sacrifice and lesion size and lesional macrophage content was determined using Pearson correlation coefficients and significance was determined using linear regression analysis. After 13 weeks, the percentage of (D) IL-10$^+$ B cells, (E) CD1d^{hi}CD5$^+$ B cells, (F) CD9$^+$ B cells and (G) Tim-1$^+$ B cells were determined in the spleen with flow cytometry. Data are shown as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$) and n = 5–8/group.

compared to levels in WT mice (Fig. 2E and F). Tim-1 itself is an important Breg marker [15,21], and as *Tim-1*$^{\Delta mucin}$ mice express Tim-1 at normal levels, albeit functionally impaired, we also observed significantly decreased Tim-1$^+$ B cells in *Tim-1*$^{\Delta mucin}$ mice compared to WT mice (Fig. 2G). These findings highlight that during atherosclerosis development, *Tim-1*$^{\Delta mucin}$ mice display an impaired IL-10 production by Bregs.

3.3. Increased viable leukocytes in *Tim-1*$^{\Delta mucin}$ mice

Deficiencies in IL-10-producing B cells have previously been shown to induce an activated immune phenotype [15]. We examined if the development of atherosclerosis in *Tim-1*$^{\Delta mucin}$ mice led to a similar activation of the immune system. We first measured spleen weight (Fig. 3A) and the total number of spleen cells (Fig. 3B) and did not find differences between WT and *Tim-1*$^{\Delta mucin}$ mice. Tim-1 is involved in the recognition of apoptotic cells [22] and flow cytometry experiments displayed a consistent increase in the percentage of viable white blood cells both in the spleen and circulation of *Tim-1*$^{\Delta mucin}$ mice compared to WT mice (Fig. 3C). We further examined if specific leukocyte populations were increased, which revealed that the number of splenic CD4$^+$ T cells and CD19$^+$ B cells were significantly increased, while CD11b$^+$ leukocytes and dendritic cells (DCs) were markedly decreased in the spleen of *Tim-1*$^{\Delta mucin}$ mice compared to WT mice (Fig. 3D). Analysis of circulating cells revealed a similar phenotype with increased CD19$^+$ B cells and a percentage of circulating monocytes (Fig. 3E). Despite reduced anti-inflammatory Bregs (Fig. 2), elevated pro-atherogenic follicular (FO) B cells contributed to the increase in CD19$^+$ B cells found in spleens of *Tim-1*$^{\Delta mucin}$ mice, while marginal zone (MZ) and B1a B cells remained unaffected (Fig. 3F). Notably, B1a B cells can also be a source of IL-10 but as shown in Supplementary Fig. 3A these cells were not responsible for the reduced IL-10$^+$ B cells upon Tim-1 deficiency. In addition to the decrease in splenic DCs (Fig. 3E), a trend towards reduced mature MHCII^{hi} DCs was found in *Tim-1*$^{\Delta mucin}$ mice (Fig. 3G). However, MHCII expression per DC did not differ (Supplementary Fig. 3B).

3.4. CD4$^+$ T cell differentiation but not activation is affected in *Tim-1*$^{\Delta mucin}$ mice

To investigate if the increase in splenic CD4$^+$ T cells was due to enhanced CD4$^+$ T cell activation, we first assessed the expression of CD69 and the proliferation marker Ki-67 in CD4$^+$ T cells. This, however, did not reveal any (statistical significant) differences between the different mice phenotypes (Supplementary Fig. 4). Next, we isolated splenocytes and performed an *ex vivo* proliferation assay. Irrespective of the stimulus used (i.e. oxLDL or anti-CD3/anti-CD28), splenocytes from WT and *Tim-1*$^{\Delta mucin}$ mice showed a comparable proliferation (Supplementary Fig. 5). Similarly, levels of IL-2 in the supernatants of anti-CD3/anti-CD28 stimulated splenocytes did not differ between WT and *Tim-1*$^{\Delta mucin}$ mice (Supplementary Fig. 5). These data suggest that the activation of CD4$^+$ T cells in *Tim-1*$^{\Delta mucin}$ mice was not affected during atherosclerosis development. We subsequently tested whether the differentiation of CD4$^+$ T cells was altered, since Tim-1 appears to be primarily involved in Th2 responses [6,23]. We noticed that spleens of *Tim-1*$^{\Delta mucin}$ mice contained significantly more naïve CD4$^+$ T cells

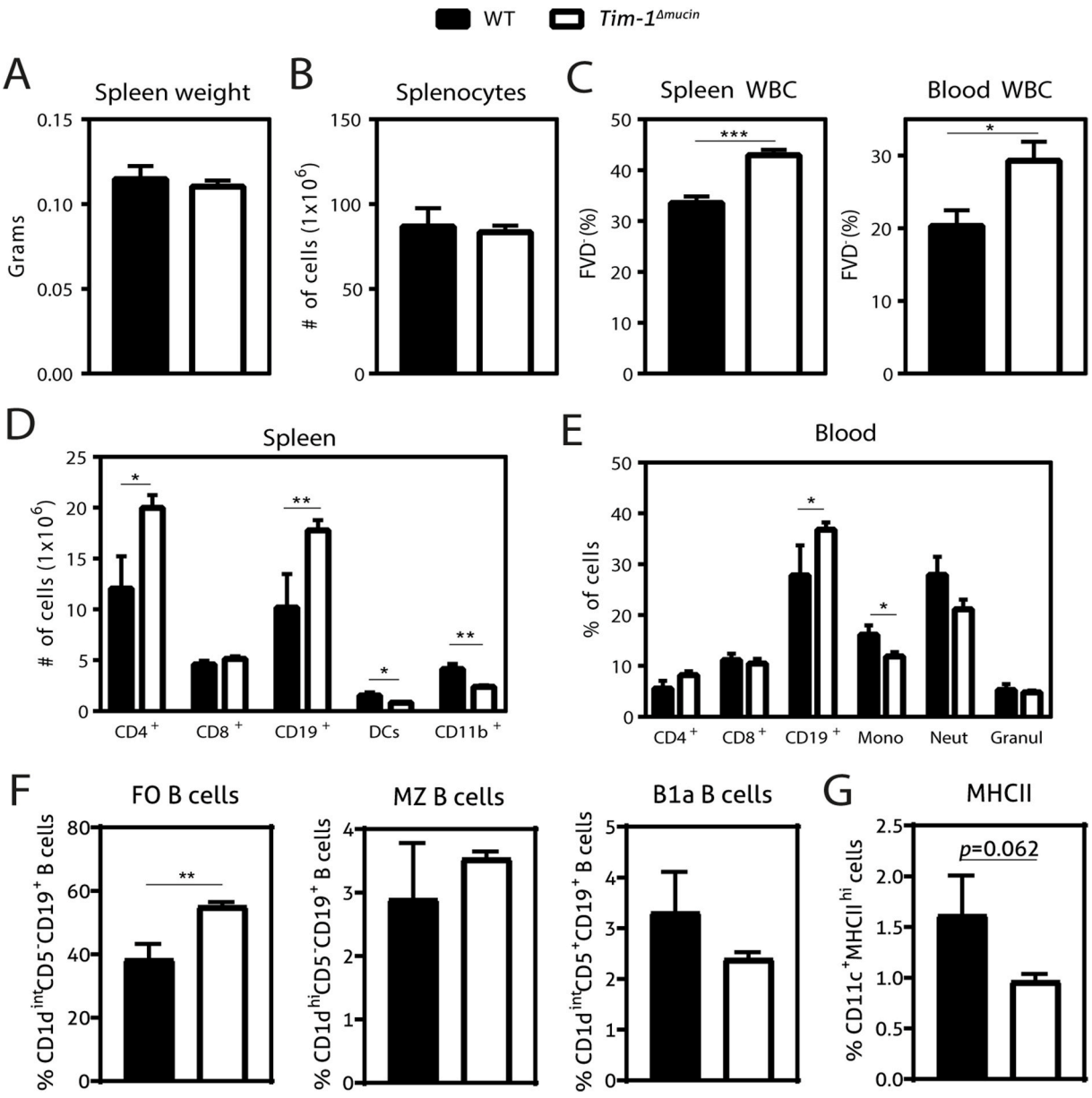

Fig. 3. Increased viable leukocytes in *Tim-1*$^{\Delta mucin}$ mice.
At sacrifice, (A) spleens of C57BL/6 (WT) and Tim-1-mucin domain-deficient (*Tim-1*$^{\Delta mucin}$) mice were weighed and (B) the total number of splenocytes were analyzed with a Sysmex hematolyzer. Using flow cytometry the percentage white bloods cells negative for Fixable Viability Dye (FVD) were assessed (C). Specific leukocyte populations in the (D) spleen and (E) circulation were determined. Splenic (F) CD1d^{int}CD5^{-} follicular (FO) B cells, CD1d^{hi}CD5^{-} marginal zone (MZ) B cells and CD1d^{int}CD5^{+} B1a B cells and (G) CD11c $^{+}$ MHCII^{hi} cells were measured with flow cytometry. Data are shown as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) and n = 5–8/group.

compared to spleens of WT mice (Fig. 4A), while no difference was found in the number of effector memory CD4^{+} T cells. More detailed analysis of the CD4^{+} T cell subsets showed that *Tim-1*$^{\Delta mucin}$ mice demonstrated a significant decrease in the number of Th1 and Th17 cells compared to WT mice, with an even more remarkable decrease in the number of Th2 cells (Fig. 4B). Subsequently, we used a 5 h *ex vivo* stimulation with PMA and ionomycin to determine cytokine production by CD4^{+} T cells from WT and *Tim-1*$^{\Delta mucin}$ mice. While we did not find any differences between *Tim-1*$^{\Delta mucin}$ and WT mice in IFNγ, IL-4 or IL-10 production with flow cytometry (Supplementary Fig. 6), we noticed a significant shift in the IFNγ/IL-4 ratio indicating a reduced Th2-response (Fig. 4C). We wanted to further investigate this effect and measured additional cytokines in the culture supernatants of our *ex vivo* proliferation assay. In line with a reduced Th2-response, we did not observe any differences in Th1-, Th17- and Treg-associated cytokines in culture supernatants (Supplementary Fig. 7), however we observed a large decrease in the Th2-associated cytokines IL-4 and IL-5 in culture supernatants from *Tim-1*$^{\Delta mucin}$ splenocytes compared to WT splenocytes (Fig. 4D). These data indicate that impaired Tim-1 signaling significantly decreased the differentiation of naïve CD4^{+} T cells towards Th2 cells.

3.5. Humoral immunity is unaffected in *Tim-1*$^{\Delta mucin}$ mice

Isotype switching of B cells is strongly influenced by local cytokine production and given the increased number of splenic B cells and decrease in Th2 cells, we wondered if the humoral immunity was altered in *Tim-1*$^{\Delta mucin}$ mice. We measured total IgG1 (Fig. 5A) and IgG2c (Fig. 5B), which did not reveal any differences between the two groups. Given the shift we observed in the Th1/Th2 ratio, we examined the

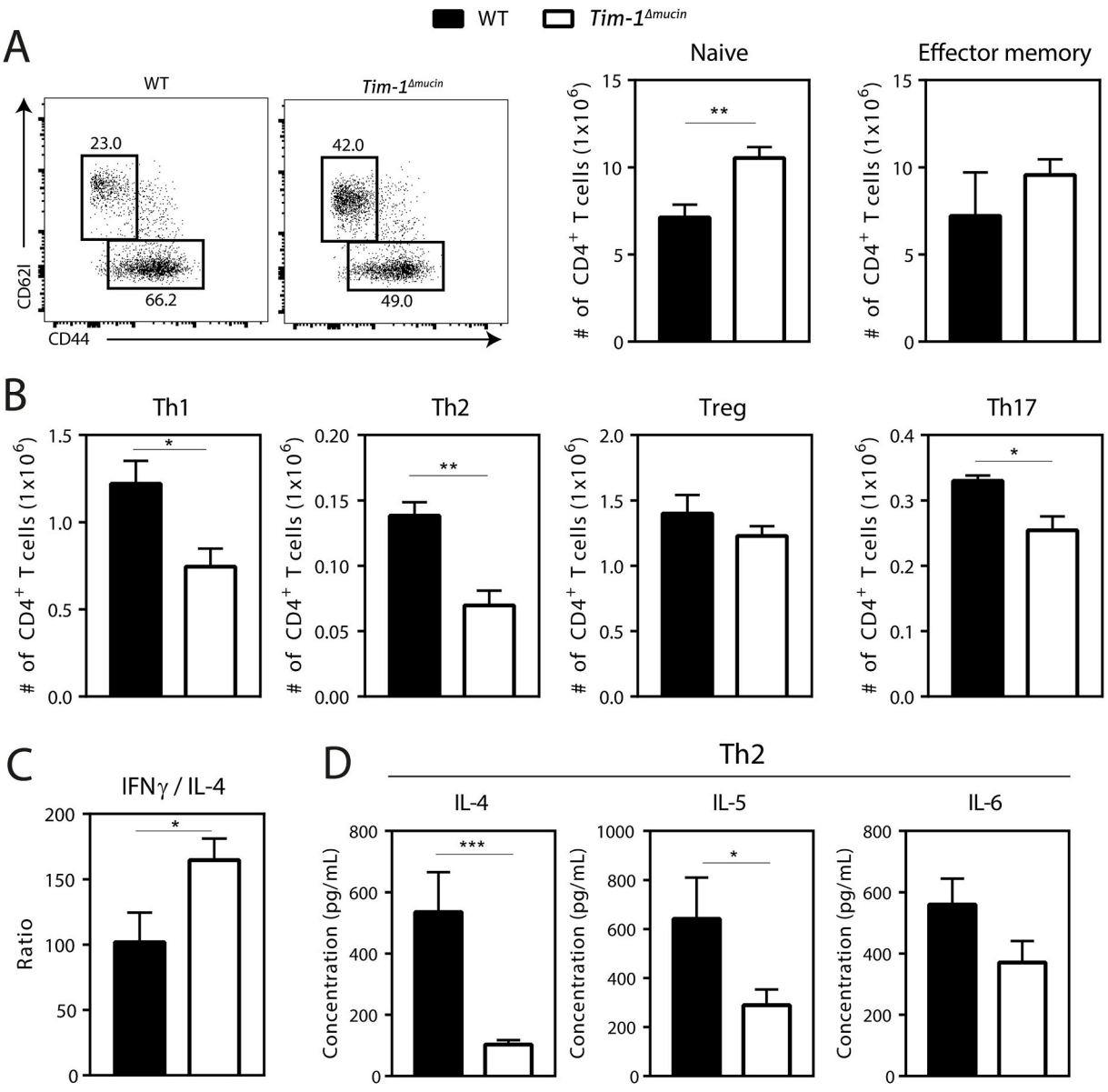

Fig. 4. CD4$^+$ T cell response in *Tim-1*$^{\Delta mucin}$ mice.
At sacrifice, splenocytes of C57BL/6 (WT) and Tim-1-mucin domain-deficient (*Tim-1*$^{\Delta mucin}$) mice were analyzed with flow cytometry to determine the number of (A) naïve (CD62l$^+$ CD44^{lo}) and effector memory (CD62l$^-$CD44^{hi}) CD4$^+$ T cells and (B) Th1 (T-bet$^+$), Th2 (Gata-3$^+$), Treg (CD25$^+$FoxP3$^+$), Th17 (RORγt$^+$) CD4$^+$ T cells. (C) Single cell suspensions of splenocytes were stimulated for 5 h with PMA, ionomycin and Brefeldin A after which intracellular IFNγ and IL-4 was measured and the ratio between IFNγ$^+$ and IL-4$^+$ CD4$^+$ T cells was determined. (D) Splenocytes were cultured in RPMI medium and stimulated with anti-CD3 (1.25 μg/ml) and anti-CD28 (1.25 μg/ml). After 72 h, supernatant was harvested and analyzed with a multiplex assay for Th2-associated cytokines. Data are shown as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) and n = 5–8/group.

IgG2c/IgG1 ratio and found a slight but non-significant trend towards an increased ratio (Fig. 5C). Similarly, we did not find any differences in total oxLDL-specific IgG levels (Fig. 5D). It has recently been shown that treatment of mice with the RMT1-10 anti-Tim-1 antibody resulted in increased peritoneal B1 cells and a concomitant increase in atheroprotective IgM antibodies [8]. In contrast, defective Tim-1 signaling did not influence peritoneal B1 cells numbers (Fig. 5E), total IgM levels (Fig. 5F) or oxLDL-specific IgM levels (Fig. 5G). Taken together, these data indicate that the humoral immunity was unaffected in *Tim-1*$^{\Delta mucin}$ mice.

4. Discussion

Tim-1 has been associated with a broad number of inflammatory disorders. We and others have previously investigated the role of Tim-1 in atherosclerosis using anti-Tim-1 antibodies [6,8]. However, due to the complex nature of Tim-1 signaling these studies demonstrated contradicting results. It is clear that Tim-1 signaling is highly dependent on binding avidity, which varies with different anti-Tim-1 antibody clones. Here, we circumvented the antibody-associated difficulties by using *Tim-1*$^{\Delta mucin}$ mice and now show that Tim-1 has an overall protective effect during atherosclerosis development.

A previous study with *Tim-1*$^{\Delta mucin}$ mice revealed that up to 6 months of age these mice did not develop any overt auto-immune responses, but already showed a mild defect in B cell-derived IL-10 production [15]. With increasing age, or when crossbred onto an autoimmune genetic background, the Breg deficiency was exacerbated and resulted in concomitant increased numbers of B and T cells. In this study, we show that the development of atherosclerosis in young *Tim-1*$^{\Delta mucin}$ mice (<6 months) was accompanied by profound impairment in B cell associated IL-10 production. Similarly, we observed leukocytosis in the spleen and

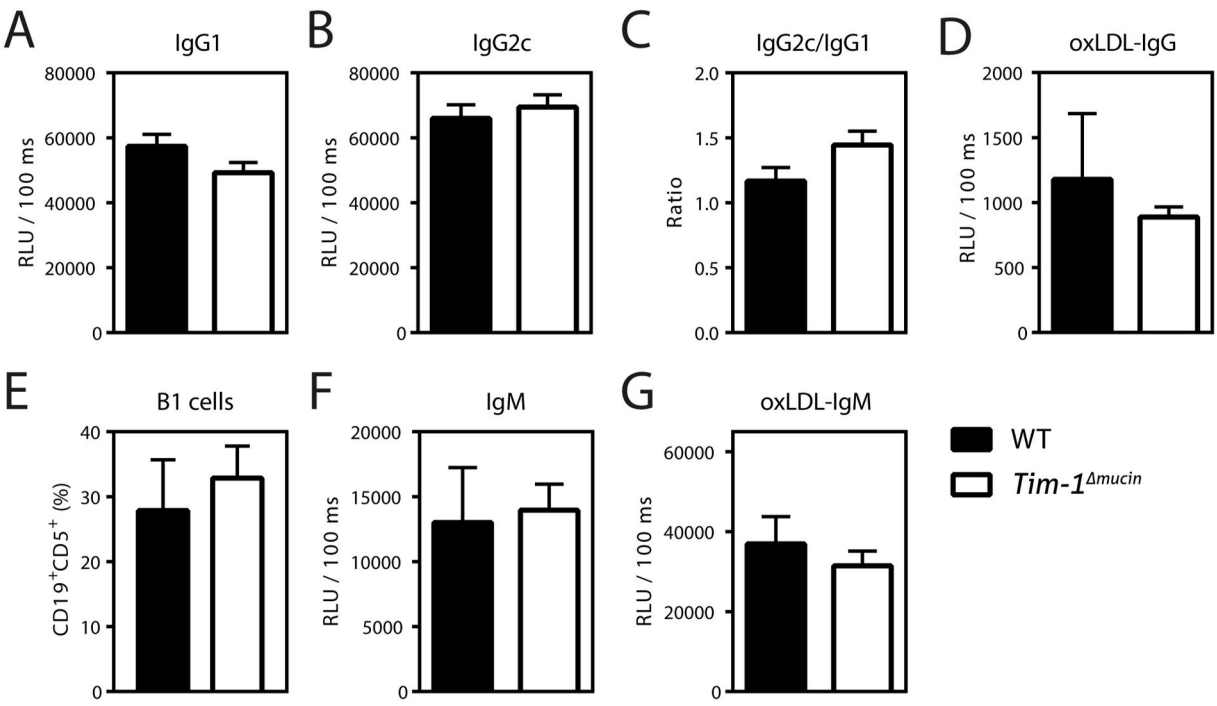

Fig. 5. The humoral immunity is unaffected in *Tim-1*$^{\Delta mucin}$ mice.
At sacrifice, serum from C57BL/6 (WT) and Tim-1-mucin domain-deficient (*Tim-1*$^{\Delta mucin}$) mice was analyzed for total (A) IgG1, (B) IgG2c and (F) IgM levels. Serum titers of (D) IgG and (G) specific for oxidized low-density lipoprotein (oxLDL) were measured. (C) Quantification of the IgG2c/IgG1 ratio. Flow cytometry was used to (E) quantify CD19^{+}CD5^{+} B1 cells in the peritoneal cavity. Data are shown as mean ± SEM and n = 5–8/group.

circulation of *Tim-1*$^{\Delta mucin}$ mice, with primarily an increase in B cells and CD4^{+} T cells. A major difference with the study of Xiao et al., however, is our observation of increased naïve but not differentiated CD4^{+} T cells, while they observed increased CD4^{+} T cell activation and differentiation in *Tim-1*$^{\Delta mucin}$ mice [15]. They demonstrated that *Tim-1*$^{\Delta mucin}$ mice crossbred with *Fas*-mutant *lpr* mice showed marked autoimmune responses, including enlarged lymphoid tissues, and hyperactive B and T cells. A potential reason for this contrast is that Tim-1 expression is highly dynamic and depending on the context and immune status its resulting effect can be either positive or negative coinhibition. *Fas*-deficient *lpr* mice on a C57BL/6 background already develop systemic autoimmunity [24], while atherosclerosis induces a much milder autoimmune response. This difference in immune status between the disease models could have influenced the CD4^{+} T cell status in response to a lack in Tim-1 signaling. Additionally, we found that the number of splenic DCs was significantly reduced in our atherosclerotic *Tim-1*$^{\Delta mucin}$ mice with a concomitant trend towards a reduced percentage of mature MHCII^{hi} DCs. Tim-1 is constitutively expressed on DCs and Tim-1 signaling on DCs is associated with increased CD4^{+} T cell effector responses [25]. Thus the reduced number, maturation state and absence of Tim-1 on DCs could also have contributed to the decreased effector CD4^{+} T cells in this study.

The two previous studies investigating the role of Tim-1 in atherosclerosis demonstrated contrasting results [6,8]. While we previously showed that antibody blockade of Tim-1 was associated with a shift in the Th1/Th2 balance and exacerbated atherosclerosis, Hosseini et al. demonstrated that Tim-1 inhibition resulted in atheroprotective IgM-producing B1a and IL-10^{+} B cells [8]. In this study we investigated atherosclerosis in Tim-1 deficient mice and found a combination of these effects. It has been shown that Tim-1 is predominantly expressed on Th2 cells, while expression is low on Th1 cells [26]. In addition, Tim-1 is frequently associated with allergic diseases which are mainly Th2-driven [3]. In line with these findings, Th2 cells and Th2-associated cytokines, which previously have been associated with a reduced risk of CVD [27], were decreased in *Tim-1*$^{\Delta mucin}$ mice. In contrast, Th1- and Th17-associated cytokines were similar in *Tim-1*$^{\Delta mucin}$ and WT mice, resulting in a significant switch in the Th1/Th2 balance. While our observation of increased naïve CD4^{+} T cells would be expected to reduce atherosclerosis, atherosclerosis is primarily a Th1-driven disease and skewing towards a Th1-dependent immune response in *Tim-1*$^{\Delta mucin}$ mice will most likely have significantly contributed to the increased atherosclerosis development in these mice. Locally in the aortic root, we did not observe differences in CD4^{+} T cell numbers in *Tim-1*$^{\Delta mucin}$ mice, but CD8^{+} T cells were reduced. This could be related to increased macrophages present in the lesions of *Tim-1*$^{\Delta mucin}$ mice as previously it has been reported that macrophages and CD8^{+} T cells are inversely correlated [28]. However, we did not observe a correlation between these cells in our experiment (data not shown) and we also did not find any differences in CD8^{+} T cell content in circulation or spleen of *Tim-1*$^{\Delta mucin}$ mice compared to controls. Nonetheless, a more detailed analysis regarding T cell subsets locally in the lesion with flow cytometry may shed more light on the underlying mechanisms involved.

Besides the effects on CD4^{+} T cells, we also observed significant differences in IL-10^{+} B cells in *Tim-1*$^{\Delta mucin}$ mice which inversely correlated with lesion size. It is well-known that Tim-1 has a prominent role in the maintenance and generation of IL-10^{+} B cells [8,10,21]. In other auto-immune disorders, IL-10^{+} B cells have shown great therapeutic potential, but their role in atherosclerosis is not clearly defined [29–31]. The work of Hosseini et al., however, showed that increased IL-10^{+} and Tim-1^{+} B cells after treatment with an partial agonistic Tim-1 antibody was associated with decreased atherosclerosis [8], indicating that the strong decrease in IL-10^{+} B cells and Breg subsets we found in *Tim-1*$^{\Delta mucin}$ mice could contribute to the exacerbated lesion development. Moreover, we also observed elevated splenic follicular B cells in *Tim-1*$^{\Delta mucin}$ mice, which as previously shown by Tay et al. can contribute to atherosclerosis progression as well [32]. Taken together, our work identified that mice completely deficient in effective Tim-1 signaling both showed an imbalance in the Th1/Th2 ratio as well as impaired numbers of IL-10^{+} B cells and elevated FO B cells during atherosclerosis development.

Previously, we reported that Tim-1 inhibition resulted in increased IgG1 antibodies [6] and others demonstrated that Tim-1 inhibition resulted in increased IgM production by B1a cells [8], while *Tim-1*$^{\Delta mucin}$ mice do not display any alterations in antibody levels. One explanation for these discrepancies might lie in the use of different experimental atherosclerosis models. While we previously used low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mice on a WTD, Hosseini et al. investigated atherosclerosis development in apolipoprotein E-deficient mice. In the current study, we introduced atherosclerosis in *Tim-1*$^{\Delta mucin}$ mice by a Pcsk9 adeno-associated virus yielding elevated Ldl serum levels as a consequence of Pcsk9-mediated downregulation of the Ldl receptor. As alluded to above, the expression and function of Tim-1 is highly dynamic and therefore also susceptible for the underlying disease pathology. This is also illustrated by the fact that Tim-1 does not appear to have one unifying effect in inflammatory disorders [30]. Hence the subtle differences in experimental atherosclerosis models might have added to the observed differences found in these three studies. Similarly, during the entire experiment mice of both groups gained weight, but from week 8–13 this increase was less pronounced in *Tim-1*$^{\Delta mucin}$ mice. No body weight alterations have been observed in previous studies investigating the *Tim-1*$^{\Delta mucin}$ mice or the Tim-1 pathway in atherosclerosis [6,8,15]. Although we cannot exclude that Tim-1 affects metabolic pathways, the reduced weight gain did not lead to reduced atherosclerosis. In contrast, *Tim-1*$^{\Delta mucin}$ mice showed aggravated atherosclerosis.

Finally, as Tim-1 can aid in removal of apoptotic cells, we were surprised to find elevated viability of cells in Tim-1 deficient mice. It is however described that absence of Tim-1 can lead to hyperactive B and T cells [15] and we do observe leukocytosis in the spleen and circulation with elevated FO B cells and naïve T cells. Possibly, this contributes to the increased cell viability in *Tim-1*$^{\Delta mucin}$ mice but more research is needed to support this.

In summary, this study has shed more light on the role of Tim-1 during atherosclerosis development. We show here that *Tim-1*$^{\Delta mucin}$ mice display a profound impairment of IL-10^{+} B cells, elevated follicular B cells and an imbalance in the Th1/Th2 ratio, which is associated with exacerbated lesion development. This work, combined with earlier data in mice, represents a firm foundation for future studies that could focus on elucidating the role of TIM-1 in human cardiovascular disease.

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CRediT authorship contribution statement

Hidde Douna: Investigation, Writing – original draft. **Virginia Smit:** Investigation, Writing – review & editing. **Gijs H.M. van Puijvelde:** Investigation. **Mate G. Kiss:** Investigation. **Christoph J. Binder:** Resources, Writing – review & editing. **Ilze Bot:** Investigation, Writing – review & editing. **Vijay K. Kuchroo:** Resources. **Andrew H. Lichtman:** Writing – review & editing. **Johan Kuiper:** Writing – review & editing. **Amanda C. Foks:** Conceptualization, Investigation, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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