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B cell modulation in atherosclerosis

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BTLA stimulation protects against atherosclerosis by regulating follicular B cells

Submitted

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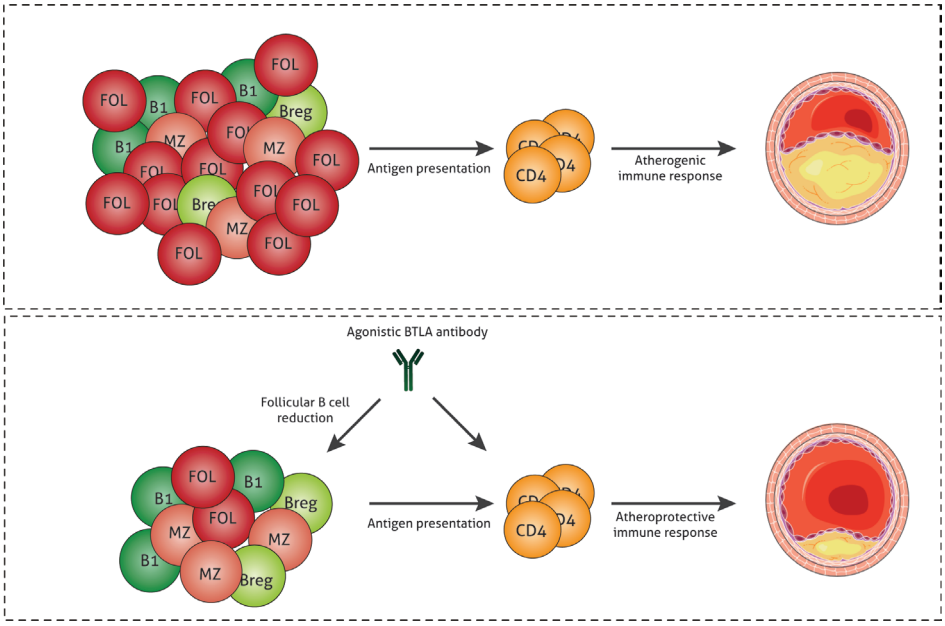
Abstract

Aims: The immune system is strongly involved in atherosclerosis and immune regulation generally leads to attenuated atherosclerosis. B and T lymphocyte attenuator (BTLA) is a novel co-receptor that negatively regulates the activation of B and T cells, however, there have been no reports of BTLA and its function in atherosclerosis or cardiovascular disease (CVD). We aimed to assess the dominant BTLA expressing leukocyte in CVD patients and to investigate whether BTLA has a functional role in experimental atherosclerosis.

Methods and results: We show that BTLA is primarily expressed on B cells in CVD patients and follicular B2 cells in low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mice. We treated *Ldlr*$^{-/-}$ mice that were fed a Western-type diet (WTD) with PBS, an isotype antibody or an agonistic BTLA antibody (3C10) for 6 weeks. We report here that the agonistic BTLA antibody significantly attenuated atherosclerosis. This was associated with a strong reduction in follicular B2 cells, while regulatory B and T cells were increased. The BTLA antibody showed similar immunomodulating effects in a progression study in which *Ldlr*$^{-/-}$ mice were fed a WTD for 10 weeks before receiving antibody treatment. Most importantly, BTLA stimulation stabilized preexisting lesions.

Conclusion: Stimulation of the BTLA pathway in *Ldlr*$^{-/-}$ mice reduces initial lesion development and increases stability of established lesions, presumably by shifting the balance between atherogenic follicular B cells and atheroprotective B cells and directing CD4^{+} T cells towards regulatory T cells. We provide the first evidence that BTLA is a very promising target for the treatment of atherosclerosis.



Graphical abstract.

Introduction

Inflammation is a key process in the development of atherosclerosis[1]. Components of both the innate and adaptive immune system contribute significantly to the pathology of atherosclerosis. The connection between these two arms of the immune system is provided by professional antigen presenting cells (APCs). Dendritic cells and macrophages are well known APCs, but nowadays the role of B cells as potent APCs is also increasingly recognized. Next to processing and presenting antigens to lymphocytes, these cells provide additional stimuli in the form of co-receptors. In fact, the activation of T cells and B cells is tightly controlled by these immune checkpoint proteins [2]. APCs have a wide-ranging variety of both stimulatory and inhibitory immune checkpoint proteins. In general, stimulatory co-receptors are found to be atherogenic, while inhibitory proteins attenuate atherosclerosis [2].

B- and T-lymphocyte attenuator (BTLA, also known as CD272) is a relatively recently described co-inhibitory receptor belonging to the immunoglobulin superfamily. It was originally identified in search for a T helper type 1 (Th1) marker [3] and it has been demonstrated that BTLA interacts with herpesvirus entry mediator (HVEM) [4]. HVEM is part of the tumor necrosis factor receptor superfamily and the BTLA-HVEM interaction is an unique example of cross-talk between the immune

checkpoint proteins of both superfamilies. Ligation of HVEM to BTLA generates an inhibitory signal which is supported by the fact that BTLA-deficient T and B cells are hyperresponsive [3,5,6]. In addition, BTLA-deficient mice show a marked phenotype of auto-immunity with increased levels of auto-antibodies, spontaneous development of autoimmune-like hepatitis [7] and increased susceptibility towards experimental autoimmune encephalomyelitis [3]. In humans, BTLA seems to function similarly since BTLA cross-linking on human T cells leads to suppressed proliferation and cytokine production [8]. Moreover, in patients with rheumatoid arthritis [9] and type 1 diabetes [10] functional polymorphisms or altered expression levels of BTLA have been found.

Hence, since its discovery as an inhibitory co-receptor, BTLA has attracted a lot of attention as a potential target for immunotherapy. While in oncology novel ways to block BTLA are explored [11], it has been shown that in autoimmune or inflammatory disorders stimulation of BTLA is a more promising option. For instance, agonistic antibodies for BTLA have been used in experimental models of Behcet's disease [12], cerebral malaria [13], graft-versus-host disease [14] and cardiac allograft rejection [15]. These data clearly indicate that the BTLA pathway is a potent option for the treatment of autoimmune disorders. Up to date there have been no reports of BTLA and if it is involved in atherosclerosis or CVD.

In the present study, we therefore aimed to characterize BTLA-expressing leukocytes in CVD patients and to investigate whether BTLA has a functional role in atherosclerosis by using a non-depleting agonistic BTLA antibody.

Methods

Human samples

Eleven anonymous atherosclerotic plaques and blood were collected post-operatively from carotid or femoral arteries endarterectomy surgeries performed in 2017 and 2018 at the Haaglanden Medical Center (HMC), Westeinde, The Hague, The Netherlands. The collection of samples was performed conform the declaration of Helsinki regarding ethical principles for medical research involving human subjects (METC registration number 17-046). All atherosclerotic samples were processed to single cell suspensions as described previously[16]. In short, cell suspensions from human atherosclerotic plaques were obtained upon digestion with collagenase IV (Gibco) and DNase (Sigma) for 30 minutes at 37°C prior to single-cell separation through a 70µm cell strainer. Red blood cells in blood samples were lysed using ACK lysis buffer. All human white blood cell populations were characterized by

flow cytometry based on the expression of the pan-leukocyte marker CD45. Gating strategies are shown where appropriate.

Animals

Female low-density lipoprotein receptor-deficient mice (*Ldlr*$^{-/-}$) were bred in house and kept under standard laboratory conditions. 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. All injections were administered i.p. in a total volume of 100 µl. During the experiments, mice were weighed, and blood samples were obtained by tail vein bleeding. At the end of 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 bled by femoral artery transection followed by perfusion with phosphate-buffered saline (PBS) through the left cardiac ventricle. All animal work was approved by the Leiden University Animal Ethics Committee and the animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

In vivo experiments

Two separate diet-induced atherosclerosis experiments were performed in this study; an atherosclerosis initiation and an atherosclerosis progression experiment. For the initiation experiment, female *Ldlr*$^{-/-}$ mice (n=11-12/group) were fed a WTD for 6 weeks while either receiving an i.p. injection of PBS, an appropriate Armenian hamster isotype antibody (Innovative Research, Novi, MI; 100 µg/injection) or a non-depleting agonistic BTLA antibody[15] (3C10; 100 µg/injection) twice a week. For the progression experiment, female *Ldlr*$^{-/-}$ mice (n=11-12/group) were first fed a WTD for 10 weeks before initiating the same treatment with an isotype antibody or an agonistic BTLA antibody for 6 weeks. At the start of the treatment, one group of mice was sacrificed as a baseline group. A third in vivo experiment was performed to investigate the direct effect of BTLA blockade on T cells. Female *Ldlr*$^{-/-}$ mice (n=5-6/group) were fed a WTD for 2 weeks while, similarly as described above, receiving i.p. injections of either PBS, isotype control, BTLA, or receiving once a week anti-CD20 (Genentech, clone 5D2; 250 µg/injection) to deplete B cells, or a combination of BTLA and anti-CD20.

Flow cytometry

For flow cytometry analysis, Fc receptors of single cell suspensions of the mediastinal lymph node, spleen, blood or peritoneum were blocked with an unconjugated antibody against CD16/CD32. Samples were then stained with a fixable viability marker

(ThermoScientific) to select live cells and anti-mouse fluorochrome-conjugated antibodies (see Online Supplementary Table 1).

For the analysis of IL-10$^+$ B cells, single cell suspensions were stimulated for 5 hours with LPS (50 µg/ml), PMA (50 ng/ml), ionomycin (500 ng/ml) and monensin (2 µM). FACS analysis was performed on a FACSCanto II (Becton Dickinson) or a Cytoflex S (Beckman Coulter) and the acquired data were analyzed using FlowJo software. Gates were set according to unstimulated controls (only treated with monensin) or to isotype and fluorescence minus one controls.

Serum measurements

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 antigen-specific antibodies were quantified by ELISA as previously described [17].

B cell culture

B cells were isolated from female *Ldlr*$^{-/-}$ mice using CD19$^+$ microbeads (Miltenyi Biotec) and cultured in complete RPMI medium in the presence of different concentrations of the agonistic BTLA antibody or recombinant murine HVEM Fc (R&D systems). After 24 hours cells were harvested and either stained using an Annexin-V Apoptosis detection kit (ThermoFisher) or used for routine flow cytometry.

B cells were also isolated from mice treated with PBS or the BTLA agonistic antibody for 2 weeks. Isolated B cells were co-cultured with isolated CD4$^+$ T cells from OT-II mice in the presence of OVA323 peptide (1 µg/ml). After 72 hours, cells were harvested and analyzed with flow cytometry.

Histology

To determine plaque 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 and two flanking sections for lesion composition. 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 with a

MOMA-2 antibody (1:1000, AbD Serotec) followed by a goat anti-rat IgG-alkaline phosphatase antibody (1:100, Sigma-Aldrich). Color development was achieved using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as enzyme substrates. The relative amount of collagen and percentage of macrophages in the lesions is expressed as the ratio between the collagen- or MOMA-2-positive and the total lesion surface area. Smooth muscle cells were stained with an α-smooth muscle cell-actin (αSMA) antibody (clone 1A4, Sigma-Aldrich) and SMC positive areas were related to the total intima surface area. The necrotic core was defined as the a-cellular, debris-rich plaque area as percentage of total plaque area. $CD4^+$ T cell (RM4-5, 1:90, ThermoFisher) and B cell (RA3-6B2, 1:100, BD Biosciences) analysis in the lesion was assessed using a rat monoclonal antibody and a secondary rabbit anti-rat antibody (BA-4001, Vector). Followed by the Vectastain ABC kit (PK-4000, Vector) and color was developed using the ImmPact Novared kit (Vector). Sections where no primary antibody was used were taken as negative controls. All slides were analyzed with a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems).

For fluorescent histology, cryosections (10 µm) of the spleen were stained with anti-mouse antibodies against CD3 (SP7) and B220 (RA3-6B2). Subsequently, sections were stained with secondary goat-anti rabbit and goat-anti rat antibodies conjugated to Alexa fluor 647 and Alexa fluor 488 (Abcam). Nuclei were visualized using the Fluoroshield mounting medium containing DAPI. Sections were captured using a Nikon TiE 2000 confocal microscope with a 20x plan apochromat objective and analyzed with Nis Elements version 4.3.

Statistics

All data are expressed as mean ± SEM. Data were tested for significance using a Student's t-test for two normally distributed groups. Data from three groups or more were analyzed by an ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test. Data from experiments with 2 or more variables were analyzed by a two-way ANOVA test followed by a Sidak post hoc test. Probability values of $p < 0.05$ were considered significant. All statistical analysis was performed using GraphPad Prism 7.0.

Results

B cells from CVD patients display strong BTLA expression

There is currently no data available regarding BTLA in patients with CVD. Hence, we obtained blood and lesions from patients that underwent an endarterectomy to assess the expression of BTLA on leukocytes. We found that almost 90% of the circulating $CD19^{+}CD20^{+}$ B cells expressed high levels of BTLA, while $CD3^{+}$ T cells (8.5%) and monocytes (5.2%) showed a much more moderate expression and granulocytes expressed only minimal levels of BTLA (Online Figure I, Figure 1A and B). Within the lesion, the greater majority of leukocytes, including B cells, do not express BTLA. This is partly caused by a difference in B cell subsets that reside in the lesion compared to blood (Figure 1B). The circulating B cell population is mostly composed of naïve B cells that express the highest levels of BTLA, whereas in the lesion we mainly found effector B cell subtypes that have lower BTLA expression. These data suggest that BTLA is most abundantly expressed on circulating B cells in patients with CVD, while this expression seems to be lost on B cells that are present in the lesion.

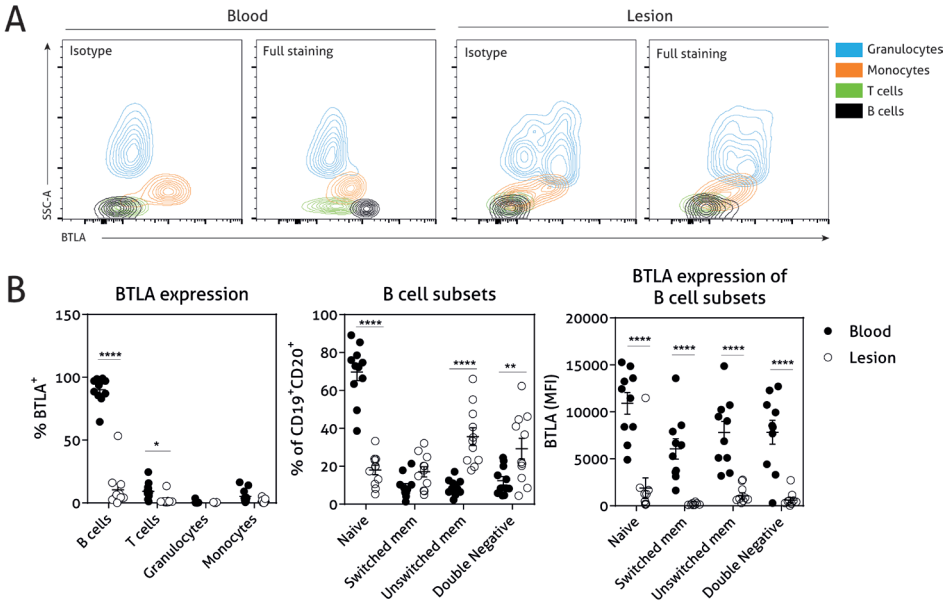


Figure 1. B cells from CVD patients display strong BTLA expression

Flow cytometry was applied on blood and lesions from CVD patients to identify BTLA expression on major leukocyte populations. **(A)** Flow charts of BTLA expression by major leukocyte populations. **(B)** Quantification of BTLA expression by leukocyte populations and B cell subsets. A two-way ANOVA followed by a Sidak post hoc test was performed. Data are shown as mean ± SEM, n=11 (* $p<0.05$, ***$p<0.001$, **** $p<0.0001$ vs. isotype).

BTLA is predominantly expressed by follicular B2 cells in *Ldlr*$^{-/-}$ mice

Given the high expression of BTLA on peripheral B cells from CVD patients and its inhibitory role, we wanted to further explore the potential of BTLA as a therapeutic target to treat atherosclerosis. First, we characterized the expression profile of BTLA on immune cells using spleens from low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mice. Similar to human leukocytes, we found that BTLA was predominantly expressed on B cells, with relatively low expression on CD4^{+} T cells, CD8^{+} T cells and innate immune cells (Figure 2A and Online Figure II-A). Moreover, we did not find any differences in BTLA expression between CD4^{+} T helper cell subsets in contrast to previous work [3] (Online Figure II-B). Interestingly, we again discovered a significant difference in BTLA expression between B cell subsets, with conventional follicular B2 cells being the most dominant BTLA-expressing leukocyte (Figure 2B).

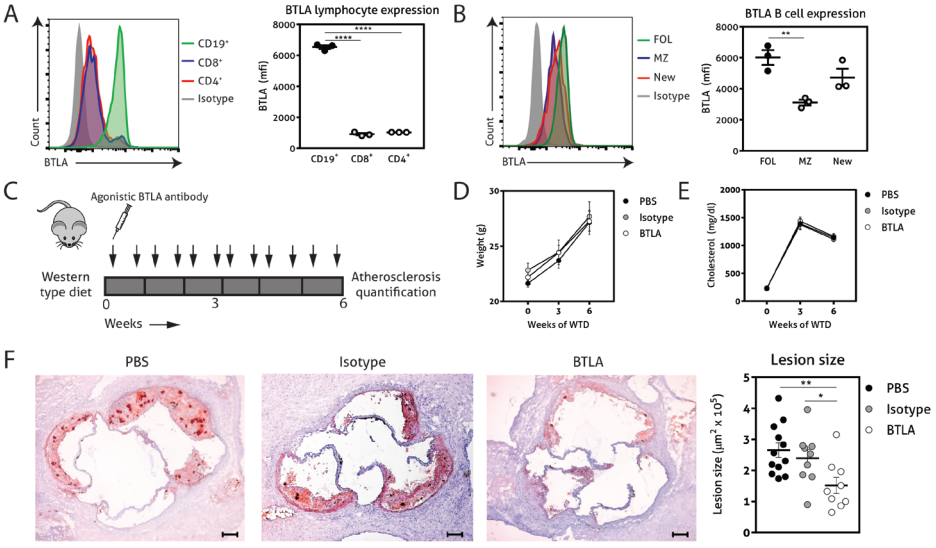


Figure 2. Activation of BTLA reduces initial atherosclerosis.
(A) Flow overlays and quantification of BTLA expression in leukocyte populations and **(B)** specific B cell subsets of female *Ldlr*$^{-/-}$ mice; follicular (CD21^{int}CD23^{+}); marginal zone (CD21^{hi}CD23^{-}) and newly formed (CD23^{lo}CD21^{lo}). **(C)** Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western-type diet. **(D)** Mice were weighed and **(E)** serum cholesterol levels were determined. **(F)** Oil-Red-O and hematoxylin staining in aortic root sections and lesion size quantification. Scale bars are 100 µm. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. Data are shown as mean ± SEM, n=3 (A/B) and n=9/12 (C-F) (** p<0.01, **** p<0.0001).

BTLA activation reduces initial atherosclerosis

The role of B cells in atherosclerosis has been quite controversial and seems to be highly dependent on the B cell subset [18]. Previously, it has been reported that

follicular B cells aggravate atherosclerosis [19–21]. Since BTLA was most abundantly expressed on follicular B cells, we hypothesized that an agonistic BTLA antibody could inhibit follicular B cells in *Ldlr*$^{-/-}$ mice, leading to attenuated atherosclerosis. We therefore treated female *Ldlr*$^{-/-}$ mice twice a week with either PBS, an isotype control antibody or an agonistic BTLA antibody (3C10) for 6 weeks while being fed a Western-type diet (WTD) (Figure 2C). The 3C10 antibody has previously been described as a non-depleting agonistic BTLA antibody [15]. Treatment with the BTLA antibody did not affect body weight or total serum cholesterol levels (Figure 2D and E). Stimulation/agonism of BTLA resulted in a significant 43% reduction in lesion size in the aortic root compared to PBS-treated mice and a significant 37% reduction compared to isotype-treated mice (Figure 2F). Correspondingly, we found that lesions from mice treated with the BTLA antibody predominantly consisted of macrophages and relatively low amounts of collagen (Online Figure III), illustrating an early lesion phenotype. In contrast, lesions from mice treated with PBS or the isotype control, showed a more advanced phenotype with relatively more collagen and less macrophage content (Online Figure III).

Activation of BTLA leads to strong follicular B2 cell reduction

To assess whether the reduced atherosclerosis could be attributed to altered immune functions, we analyzed circulating leukocytes. We found a major reduction in total white blood cells in mice treated with the BTLA antibody compared to PBS- or isotype-treated mice (Online Figure IV). This was primarily due to a major decrease in lymphocytes and a smaller decrease in monocytes. Since monocytes and macrophages contribute significantly to initial lesion formation, we measured monocytes and monocyte subsets with flow cytometry but we could not identify a difference between BTLA-treated mice and PBS- or isotype-treated mice (Online Figure V). The strong lymphocyte reduction was primarily due to a sharp decrease in B cells in the blood and a similar effect was found in the spleen, lymph node and peritoneum (Figure 3A, Online Figure VI-A). Moreover, analysis of the B cell lineages showed that BTLA agonism particularly led to a reduction in B2 cells and a relative increase in the percentage of B1 cells (Figure 3B, Online Figure VI-B). More specifically, we found that the percentage of follicular B2 cells decreased, while marginal zone B cells increased in mice treated with the BTLA antibody compared to mice treated with PBS or isotype control (Figure 3C, Online Figure VI-C). In line with these findings, fluorescent immunohistology on spleen cryosections revealed that BTLA stimulation led to a decrease of B cells immediately surrounding the T cell areas, which corresponds to a reduction in follicular B cells (Figure 3D).

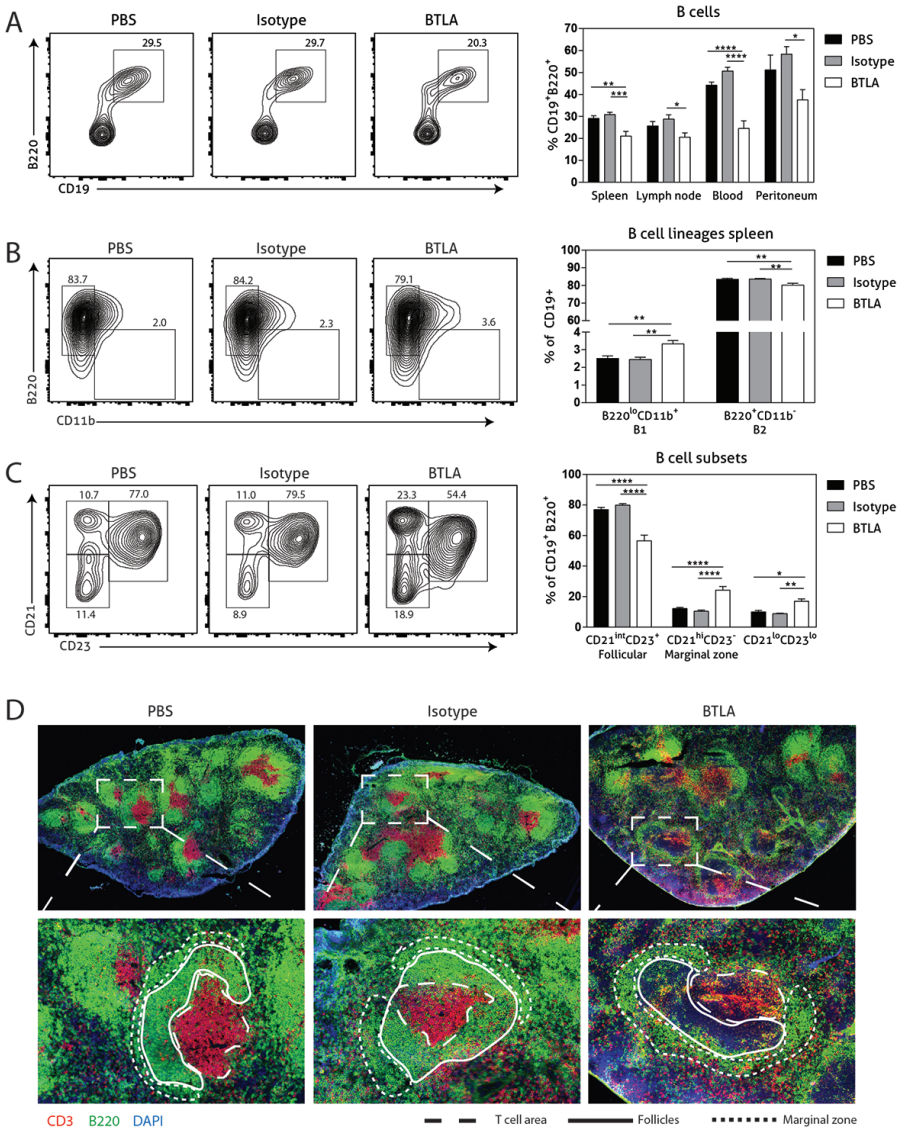


Figure 3. Activation of BTLA leads to strong follicular B2 cell reduction.
Female *Ldlr*$^{-/-}$ mice were treated twice a week with PBS, an isotype antibody or an agonistic BTLA antibody and fed a WTD for 6 weeks. **(A)** Flow charts of splenocytes and quantifications of B cells ($CD19^+B220^+$) in different organs. **(B)** Flow charts of splenocytes and quantification of B1 cells ($CD19^+CD11b^+B220^{lo}$) and B2 cells ($CD19^+CD11b^-B220^+$) in the spleen. **(C)** Flow charts of splenocytes and quantification of follicular ($CD21^{int}CD23^+$), marginal zone ($CD21^{hi}CD23^-$) and newly formed ($CD23^{lo}CD21^{lo}$) B cells in the spleen. **(D)** Representative images of spleen sections stained for CD3 (red), B220 (green) and DAPI (blue). Regions of interest are visualized as depicted at higher magnifications. Scale bars are 100 µm and 500 µm. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. Data are shown as mean ± SEM, n=11-12 (*p<0.05, ** p<0.01, ***p<0.001, **** p<0.0001).

Mechanistically, we show that BTLA agonism, similarly as BTLA-HVEM signaling, increased apoptosis and reduced activation in follicular B cells (Online Figure VII). Since follicular B cells are often involved in the humoral immunity, we next determined whether BTLA treatment reduced atherosclerosis by altering antibody responses. However, we did not find relevant differences in either total or malondialdehyde-LDL and oxidized-LDL-specific serum titer levels that could explain the ameliorated atherosclerosis (Online Figure VIII).

Activation of BTLA leads to increased regulatory B cells

Besides the conventional B1 and B2 cells, we nowadays know that there are many novel B cell subsets that can regulate the immune response [18]. Although most of these regulatory B cells (Bregs) ultimately act via interleukin-10 (IL-10), they can be identified by different extracellular markers [22], such as $CD1d^{hi}CD5^{+}$ expression [23], and T-cell immunoglobulin and mucin domain 1 (TIM-1) [24]. We found that BTLA was expressed on all of these Breg subsets (Online Figure II-C). Accordingly, *Ldlr*$^{-/-}$ mice treated with agonstic BTLA showed a very strong increase in B10 cells and TIM-1^{+} B cells in the spleen, lymph nodes and blood when compared to treatment with PBS or isotype control (Figure 4A and B). In addition, we measured the direct secretion of IL-10 by B cells and found that BTLA stimulation significantly increased the percentage of B cells that secreted IL-10 compared to the PBS or isotype control groups (Figure 4C).

Activation of BTLA leads to a protective T cell response

Treatment with the BTLA antibody resulted in a B cell pool that is highly enriched in marginal zone B cells and Breg cells, which can both inhibit the $CD4^{+}$ T cell response [22,25]. Hence, we assessed the number of lesional $CD4^{+}$ T cells and found a marked reduction in infiltrating $CD4^{+}$ T cells in BTLA-treated mice compared to PBS- or isotype-treated mice (Figure 5A). Corroborating with earlier data [21], we did not find any relevant numbers of B cells in the lesion at this stage (Online Figure IX). We thus reasoned that the $CD4^{+}$ T cell regulation had taken place peripherally as shown in earlier reports [21]. Although we did not find a difference in total splenic $CD4^{+}$ T cells, we found that BTLA-treated mice contained more effector and less naïve T cells than PBS- or isotype-treated mice (Figure 5B). More specifically, we observed an increase in regulatory (Treg) and Th17 cells in BTLA-treated mice, while no effects were found for Th1 or Th2 cells (Figure 5C). It is widely recognized that Tregs are atheroprotective [26] and Th17 cells have also been shown to protect against atherosclerosis in a setting with reduced B cells [21]. This indicates that treatment with the BTLA antibody polarized the $CD4^{+}$ T cell response *in vivo* towards a more atheroprotective reponse. The reduced activation of $CD4^{+}$ T cells in BTLA-treated mice was not dependent

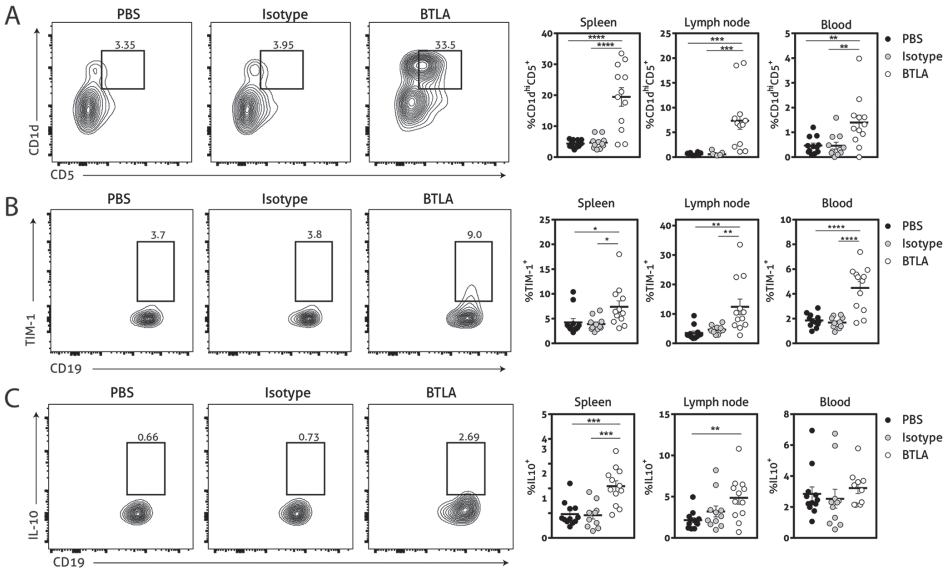


Figure 4. Activation of BTLA leads to increased regulatory B cells.
Female *Ldlr*$^{-/-}$ mice were treated twice a week with PBS, an isotype antibody or an agonistic BTLA antibody and fed a WTD for 6 weeks. Flow charts for splenocytes and quantifications of regulatory B cells are shown for **(A)** B10 cells ($CD19^+CD1d^{hi}CD5^+$), **(B)** TIM-1^+ B cells ($CD19^+$TIM-1^+) and **(C)** IL-10 secreting B cells ($CD19^+$IL-10^+). An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. Data are shown as mean ± SEM, n=11-12 (*p<0.05, ** p<0.01, ***p<0.001, **** p<0.0001).

on changes in DCs, as we did not find differences in total DCs or regulatory DCs (DEC-205^+CD8^+) between PBS-, isotype- or BTLA-treated mice (Online Figure X). To test if the altered $CD4^+$ T cell response after BTLA treatment was a direct effect of a different B cell pool, we cultured OT-II $CD4^+$ T cells with B cells from mice treated with PBS or the BTLA antibody. We found that after 72 hours of stimulation with OVA323 peptide, OT-II $CD4^+$ T cells cultured in the presence of BTLA-treated B cells, showed a marked increase in Tregs (Figure 5D). Moreover, $CD4^+$ T cells from co-cultures with BTLA-treated B cells showed a significant decrease in TNFα secretion, while IL-5 secretion was increased compared to T cells from co-cultures with PBS-treated B cells (Figure 5E). Finally, we show that the anti-inflammatory T cell response seen with BTLA agonism was not exclusively caused by changes seen in B cells, as BTLA treatment in B cell depleted mice also inhibits TNFα secretion by $CD4^+$ T cells (Online Figure XI).

Taken together, these data strongly indicate that the BTLA antibody reduced the peripheral activation of $CD4^+$ T cells, both indirectly by altering the APC function of B cells and by directly polarizing $CD4^+$ T cells, resulting in reduced lesional $CD4^+$ T cells.

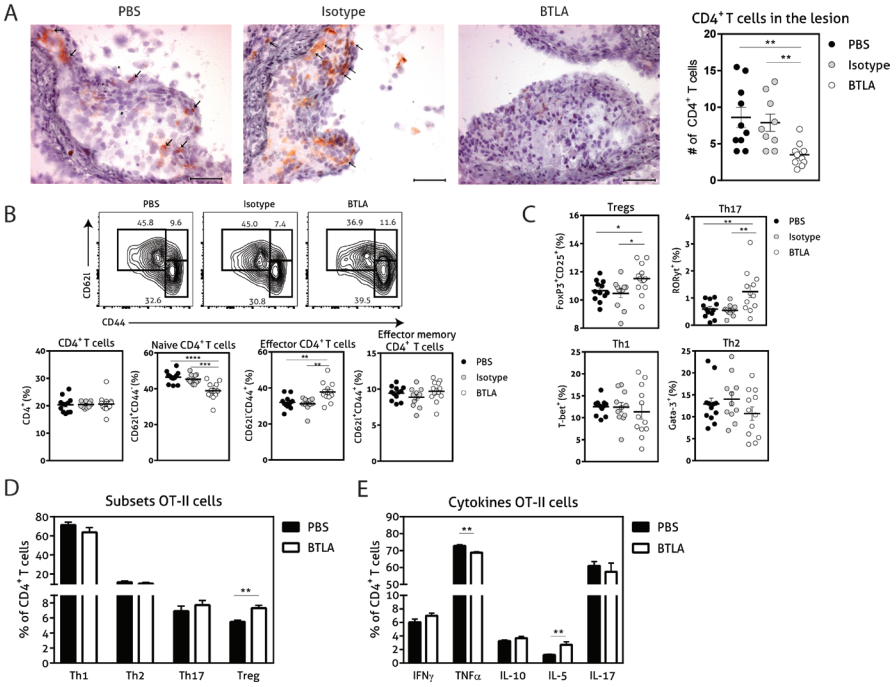


Figure 5. Activation of BTLA leads to decreased T cell infiltration and activation. Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western type diet. **(A)** Representative images of aortic root sections stained for CD4 (arrows) and hematoxyline and cell number quantification. Scale bars are 100 µm. **(B)** Flow charts and quantifications of total CD4$^+$ T cells, naive CD4$^+$ T cells (CD62l$^+$CD44$^-$), effector CD4$^+$ T cells (CD62l$^-$CD44$^+$) and effector memory CD4 T cells (CD62l$^+$CD44$^+$) in splenocytes. **(C)** Flow cytometry quantifications of regulatory T cells (FoxP3$^+$), Th17 (RORγt$^+$), Th1 (T-bet$^+$), and Th2 (Gata-3$^+$) cells in splenocytes. **(D)** B cells from mice treated with PBS or an agonistic BTLA antibody for 2 weeks were co-cultured with isolated CD4$^+$ T cells from OTII mice in the presence of OVA323 peptide (1 µg/ml). CD4$^+$ T cells were harvested after 72 hours and assessed with flow cytometry for regulatory T cells (FoxP3$^+$), Th17 (RORγt$^+$), Th1 (T-bet$^+$), and Th2 (Gata-3$^+$) cells and **(E)** for cytokine production. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. Data are shown as mean ± SEM, n=9/12 (*p<0.05, ** p<0.01, ***p<0.001, ****p<.00001). Tregs; regulatory T cells, Th; T helper cell.

Activation of BTLA leads to increased stability in established lesions

Since most CVD patients that require medication already have well-established atherosclerosis, we also investigated the effects of the BTLA antibody on pre-existing lesions. Therefore, we fed *Ldlr*$^{-/-}$ mice a WTD for 10 weeks, after which we started the agonistic BTLA or isotype treatment for 6 weeks (Figure 6A). In line with our initial atherosclerosis study, we found a highly significant reduction in total B cells in all relevant organs (Figure 6B) caused by a strong decrease in follicular B cells (Figure 6C). In contrast, IL-10$^+$ B cells (Figure 6D) and Tregs were increased (Online

Figure XII). Although we did not find differences in lesion size between isotype- or BTLA-treated mice (Figure 6E), we did observe that lesions of mice treated with the BTLA antibody contained significantly more collagen than lesions of mice treated with the isotype control (Figure 6F). Furthermore, lesional macrophages, smooth muscle cell content and necrotic core area was determined (Online Figure XIII). This shows that BTLA stimulation increased the stability of already established lesions in *Ldlr*$^{-/-}$ mice.

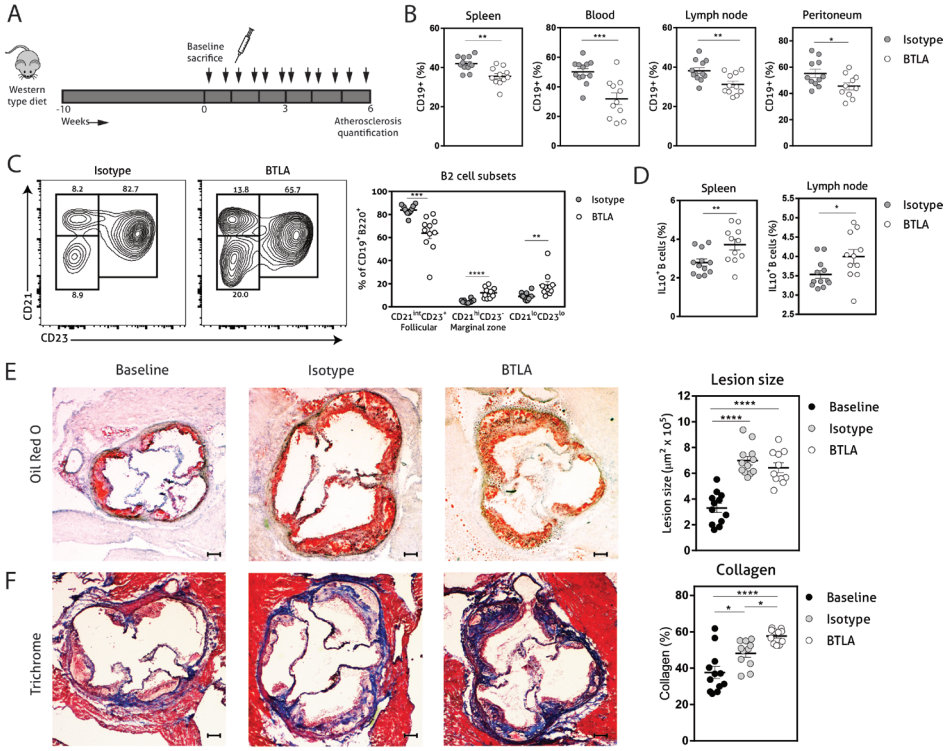


Figure 6. Activation of BTLA leads to increased stability in established lesions.
(A) Female *Ldlr*$^{-/-}$ mice were fed a WTD for 10 weeks after wich one group was sacrificed (baseline), while other mice were treated twice a week intraperitoneally with an isotype antibody or an agonistic BTLA antibody for 6 weeks. **(B)** Flow cytometry quantification of B cells (CD19^{+}) in different organs. **(C)** Flow charts of splenocytes and quantification of follicular (CD21^{int}CD23^{+}), marginal zone (CD21^{hi}CD23^{-}) and newly formed (CD23^{lo}CD21^{lo}) B cells in the spleen. **(D)** Flow cytometry quantification of regulatory B cells (CD19^{+}IL-10^{+}) in spleen and lymph node. **(E)** Oil-Red-O and hematoxylin staining in aortic root sections and lesion size quantification. **(F)** Trichrome staining in aortic root sections and collagen quantification. Scale bars are 100 µm. A Student's t-test was performed for two groups or an ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test for multiple groups. Data are shown as mean ± SEM n=11-12 (*p<0.05, ** p<0.01, ***p<0.001, **** p<0.0001).

Discussion

The discovery of immune checkpoint proteins has been a tremendous support in finding targets for immunomodulatory drugs [2]. For many of these co-receptors, therapeutic antibodies or small molecules are now being tested in experimental animal models or in clinical trials. For the treatment of atherosclerosis and CVD we still lack potent agents that target pathways other than cholesterol metabolism. There is an urgent need for such treatments, since the residual risk of CVD is still considerable even after effective cholesterol management [27]. Moreover, the recent CANTOS trial demonstrated that anti-inflammatory therapy can significantly reduce the risk of cardiovascular events [28]. In this study, we provide the first evidence that one of the newest additions to the co-receptor family, BTLA, is a very promising target for the treatment of atherosclerosis.

Up to date, most research in relation to BTLA has focused on T cells, while it is most abundantly expressed on B cells [3,6,29,30]. One apparent reason for this discrepancy could be that many inflammatory disorders are thought to be mainly T cell driven. However, with the recognition of novel B cell subsets, we gained significantly more insight into the actual contribution of B cells to inflammatory disorders. In atherosclerosis, we now know that B1 cells [19,31], marginal zone B cells [25] and regulatory B cells [32] can exert protective functions, contrary to follicular B cells that aggravate atherosclerosis [20,21]. Since follicular B cells are in the majority, complete B2 cell depletion has resulted in attenuated atherosclerosis [20,21]. Yet, these treatments deplete the complete B2 cell population which includes atheroprotective Bregs and marginal zone B cells [20,21,33]. In a side-to-side comparison study, we show that whereas anti-CD20 treatment depletes all B cells, BTLA specifically targets follicular B2 cells and can directly inhibit pro-inflammatory $CD4^+$ T cells. Secondly, the humoral immunity is compromised with complete B2 cell depletion, illustrated by reductions in both total and antigen-specific IgG and IgM antibodies [20,21]. In this study, we now describe that an agonistic antibody for BTLA specifically reduced, but not ablated, the atherogenic follicular B cells, while the atheroprotective B cell subsets were increased. Although B cell depleting antibodies are already in clinical use for other autoimmune diseases such as rheumatoid arthritis[34], we believe that BTLA stimulation might be superior to total B cell depletion in CVD.

Additionally, we have found that the humoral immunity was not affected since both total and antigen-specific IgG1, IgG2 and IgM levels remained the same. This is in line with others that showed that blockade of the HVEM/BTLA pathway also did not

alter the humoral effect following transplantation [35]. This highlights the potential benefits of using an agonistic BTLA antibody for the treatment of atherosclerosis.

Besides reducing atherogenic follicular B2 cells, we found that BTLA activation resulted in a splenic B cell population enriched in atheroprotective marginal zone B cells and Bregs. Bregs have shown great protective potential in autoimmune disorders such as collagen-induced arthritis and experimental autoimmune encephalomyelitis, primarily through their production of IL-10 [22]. Studies investigating the role of Bregs in atherosclerosis show conflicting results [18,32,36]. However, recently we found a very strong inverse correlation between atherosclerosis severity and the frequency of IL-10$^+$ B cells, and adoptive transfer of IL-10$^+$ B cells strongly reduced circulating leukocyte numbers and inflammatory monocytes in *Ldlr*$^{-/-}$ mice[37] Moreover, blockade of TIM-1, another immune checkpoint protein strongly associated with increased IL-10 producing B cells [24], leads to aggravated atherosclerosis, potentially via the blockade of TIM-1$^+$ B cells [38]. Since BTLA agonism strongly increased B10 cells and TIM-1$^+$ B cells, we believe this contributed to the reduction of atherosclerosis.

In the last years, it has been increasingly recognized that B cells have an important cellular function independent of antibody production. We found that the altered B cell population led to strongly decreased T cell activation. In addition, we showed that in both studies BTLA activation resulted in increased Tregs. During many autoimmune disorders, including atherosclerosis, Tregs become dysfunctional and are unable to curb disease [26]. Restoration of this function or expansion of Tregs has been protective in atherosclerosis [26]. Interestingly, it has previously been demonstrated that in experimental autoimmune encephalomyelitis, Bregs are able to recover the inhibitory activity of Tregs in a BTLA-dependent manner [39]. Enhanced inhibitory activity of Tregs, could also explain the decrease in T cell activation found in this study. Furthermore, we show that the BTLA antibody also directly inhibits pro-inflammatory T cells under hypercholesterolemic conditions. Overall, the expansion of Bregs and Tregs could have greatly contributed to the atheroprotective effects found with BTLA activation.

Nowadays, tremendous efforts are undertaken in the clinic to identify and treat vulnerable lesions that are prone to rupture [40]. By stimulating the BTLA pathway we were able to both reduce lesion size in an initiation study and also stabilize lesions during progression of atherosclerosis. The latter underscores that modulating the BTLA pathway presents a very promising option for clinical use. In addition, we found that in CVD patients almost 90% of all circulating B cells still express high

levels of BTLA. Despite our limited sample number, this suggests that BTLA is also an interesting and accessible target in CVD patients.

In summary, treatment with an agonistic BTLA antibody prevents atherosclerosis and stabilizes already established lesions by favorably shifting the balance between atherogenic follicular B cells and atheroprotective B cells and directing $CD4^+$ T cells towards Tregs. Our data strongly indicate that BTLA activation may be considered for the treatment of atherosclerosis.

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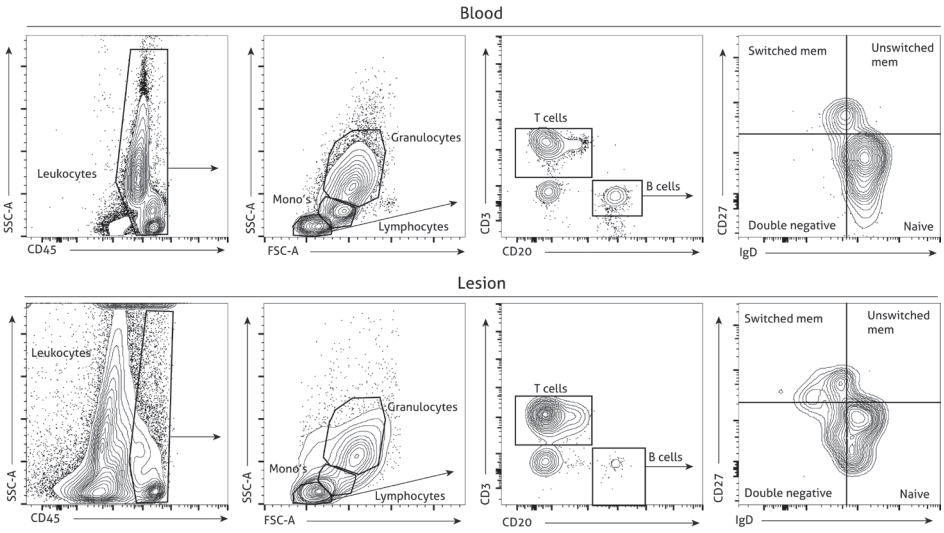
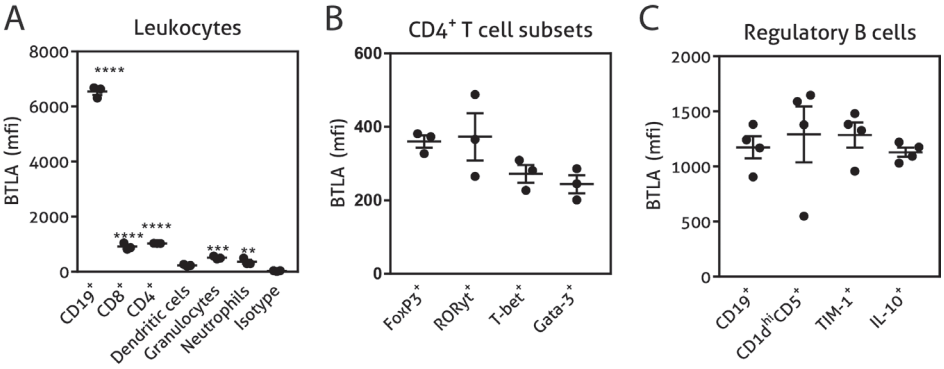
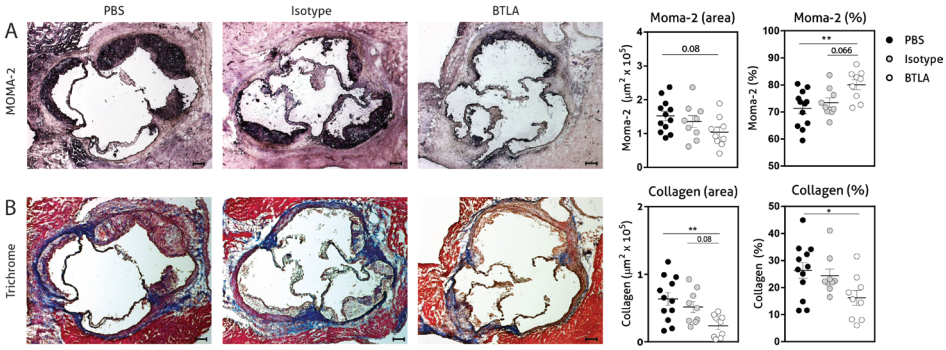


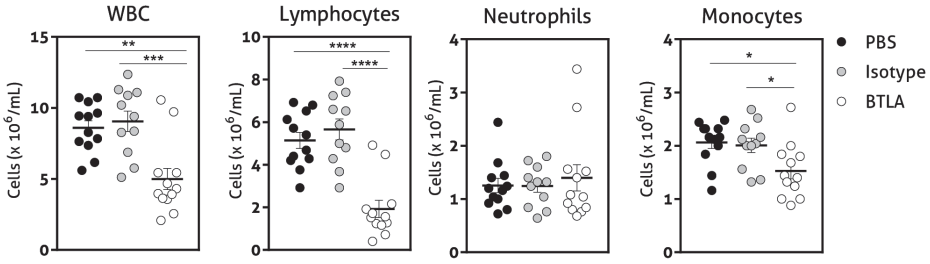
Online Figure I. **Gating strategy leukocyte population blood and lesion CVD patients.**
Flow cytometry was applied on blood and lesions from CVD patients to identify BTLA expression on major leukocyte populations. Cells were analyzed for a live/dead marker after which the indicated gating strategies were applied. B cells were also assessed for CD19 expression before subtypes were identified.



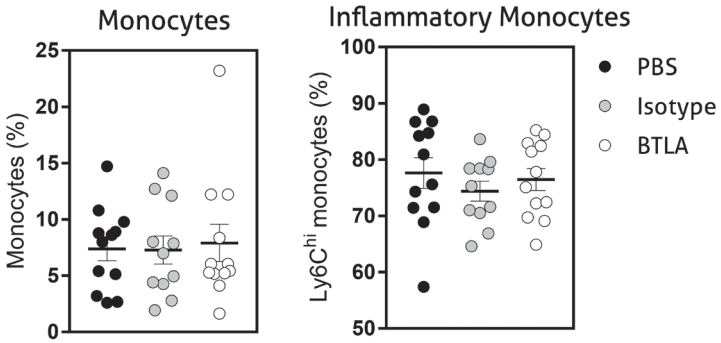
Online Figure II. **BTLA expression of CD4$^+$ T cell subsets in *Ldlr*$^{-/-}$ mice.**
(A) Quantification of BTLA expression in leukocyte populations; dendritic cells (CD19$^-$SSC^{lo}CD11c$^+$CD11b$^+$), granulocytes (SSC^{hi}CD11b$^+$), neutrophils (CD11b$^+$Ly-6G$^+$). **(B)** Quantification of BTLA expression in effector CD4$^+$ T cell subsets; Th1 (T-bet$^+$), Th2 (Gata-3$^+$), Th17 (RORγt$^+$) and Tregs (FoxP3$^+$). **(C)** Quantification of BTLA expression in regulatory B cell subsets. Data are shown as mean ± SEM, n=3. Tregs; regulatory T cells, Th; T helper cell. Data are shown as mean ± SEM. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. (** p<0.01, ***p<0.001, **** p<0.0001 vs. isotype).



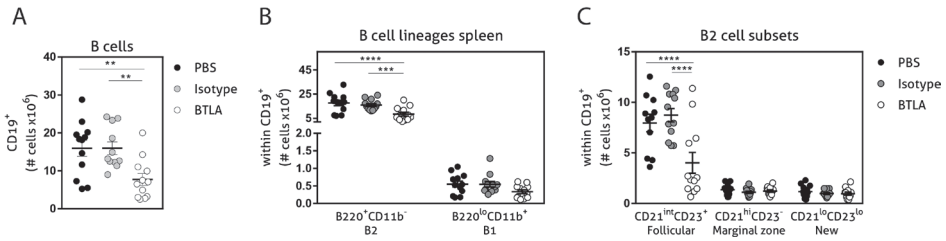
Online Figure III. **Activation of BTLA leads to an early lesion phenotype.**
Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western type diet. **(A)** MOMA-2 staining in aortic root sections with total and relative macrophage area quantification. **(B)** Trichrome staining in aortic root sections with total and relative collagen area quantification. Scale bars are 100 µm. Data are shown as mean ± SEM. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. (*p<0.05, ** p<0.01).



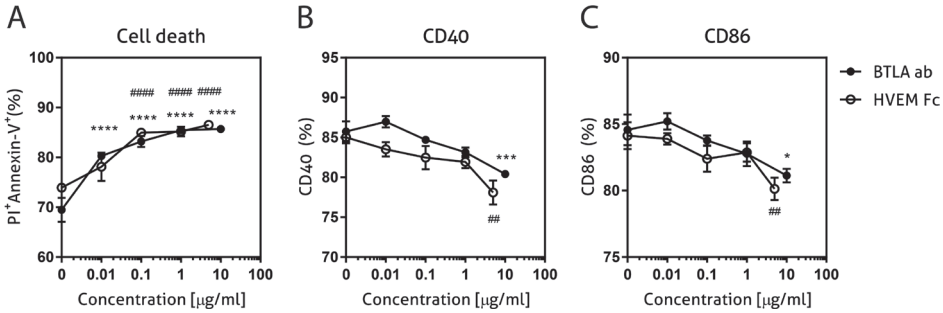
Online Figure IV. **Activation of BTLA leads to a strong reduction in circulation lymphocytes.**
Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western type diet. After sacrifice, circulating levels of total white blood cells (WBC), lymphocytes, neutrophils or monocytes were measured in full blood using a hematology analyzer. Data are shown as mean ± SEM. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. (*p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001).



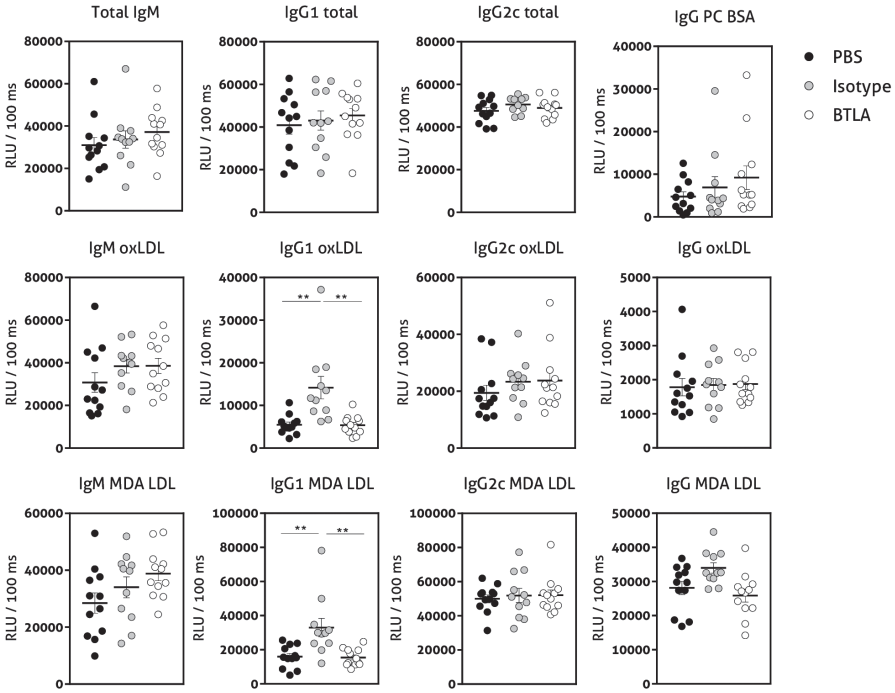
Online Figure V. **Activation of BTLA does not lead to changes in monocytes.** Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western type diet. Cirulating monocytes (B220^{-}NK1.1^{-}CD11b^{+}SSC^{int}Ly-6G^{lo}CD115^{+}) and inflammatory monocytes (B220^{-}NK1.1^{-}CD11b^{+}SSC^{int}Ly-6G^{lo}CD115^{+}Ly-6C^{hi}) were measured with flow cytometry. Data are shown as mean ± SEM.



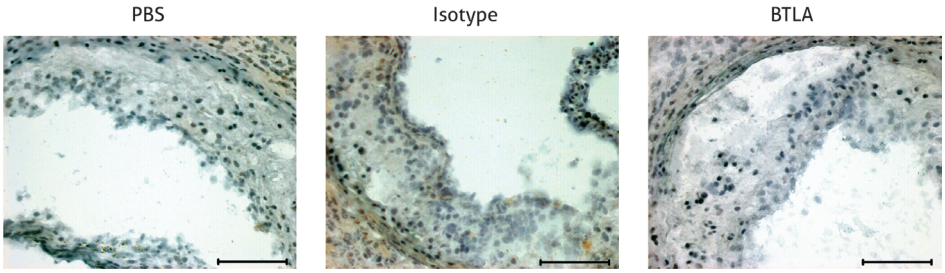
Online Figure VI. **Activation of BTLA leads to a strong reduction in abolute numbers of follicular B2 cells in the spleen.** Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western type diet. **(A)** Quantification of absolute number of B cells (CD19^{+}) in th spleen. **(B)** Quantification of absolute number of B1 cells (CD19^{+}CD11b^{+}B220^{lo}) and B2 cells (CD19^{+}CD11b^{-}B220^{+}) in the spleen. **(C)** Quantification of absolute number of follicular (CD21^{int}CD23^{+}), marginal zone (CD21^{hi}CD23^{-}) and newly formed (CD23^{lo}CD21^{lo}) B cells in the spleen. Data are shown as mean ± SEM. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. (*p<0.05, ** p<0.01).



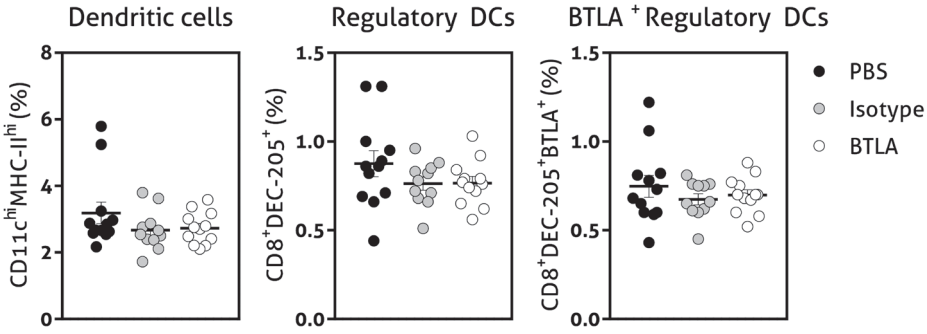
Online Figure VII. **Activation of BTLA leads increased cell death and reduced activation in B cells.** Splenic B cells were isolated from female *Ldlr*$^{-/-}$ mice with CD19^{+} microbeads and stimulated with different concentrations of an agonistic BTLA antibody or recombinant murine HVEM Fc for 24h. **(A)** Cell death was measured with propidium iodide and Annexin-V staining. B cell activation was measured using CD40 **(B)** or CD86 **(C)**. Data are shown as mean ± SEM. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. (*p<0.05, ***p<0.001, ****p<0.0001 BTLA ab vs 0 and ##p<0.01, ####p<0.0001 HVEM Fc vs 0).



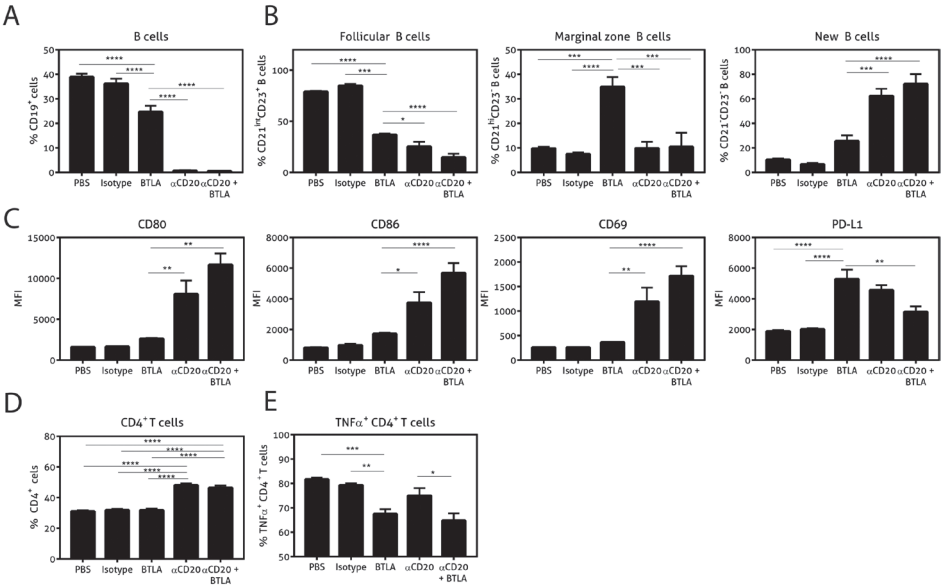
Online Figure VIII. **BTLA activation does not affect the humoral immune response.** Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western type diet. At euthanasia, serum of mice was collected in which total and antigen-specific antibodies for atherosclerosis-related antigens were determined using ELISA. Data are shown as mean ± SEM. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. (** p<0.01). oxLDL, oxidized low-density lipoprotein; MDA-LDL, malondialdehyde-modified low-density lipoprotein; BSA, bovine serum albumin; RLU relative light units; PC, phosphatidylcholine.



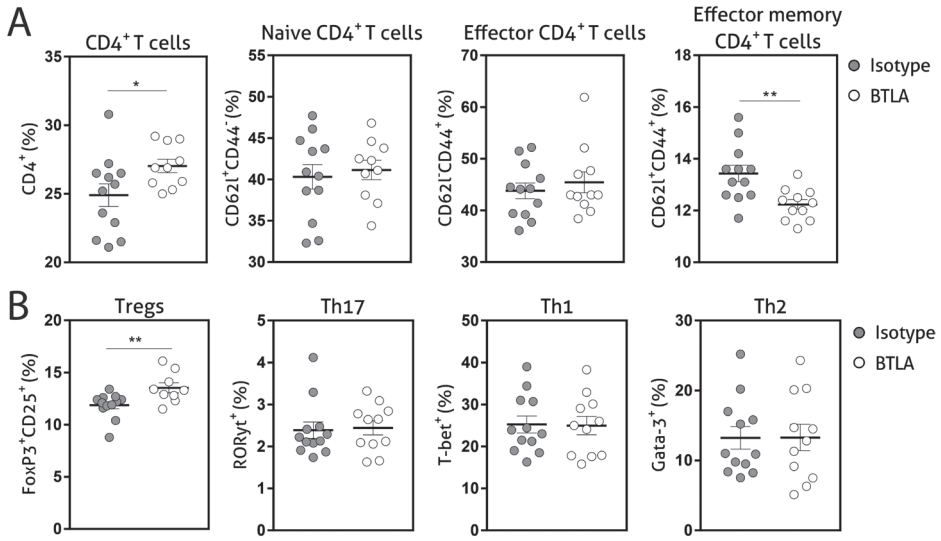
Online Figure IX. **Absence of B220^{+} cells in the aortic root.** Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western type diet. Representative images of aortic root sections stained for B220 (brown) and hematoxylin (blue) are shown. No B220^{+} cells were observed. Scale bars are 100 μm.



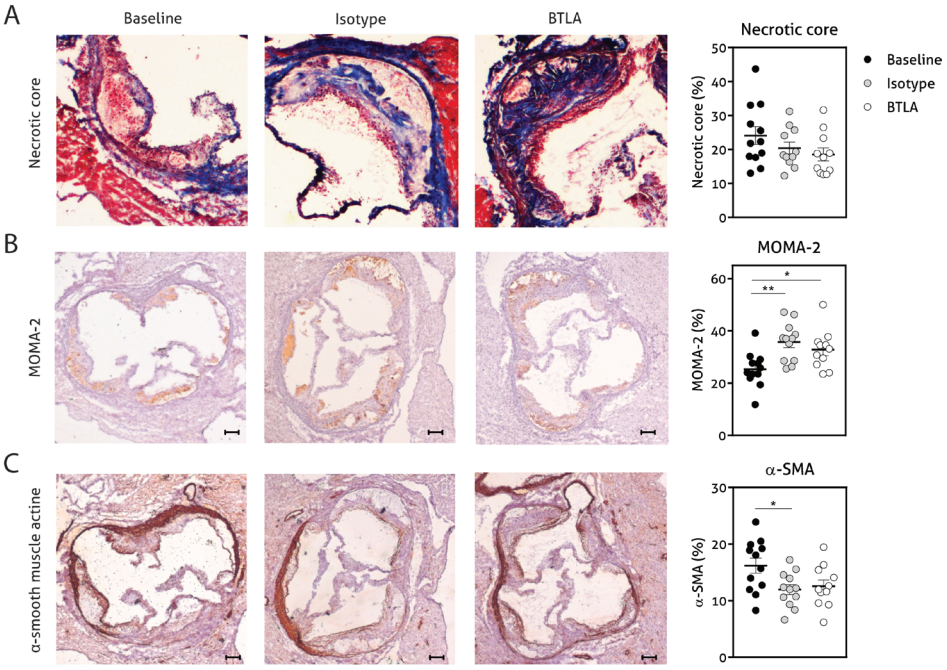
Online Figure X. **Activation of BTLA does not lead to changes in dendritic cells.** Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western type diet. Dendritic cells (CD19^{-}CD11c^{+}MHC-II^{hi}), regulatory dendritic cels (CD19^{-}CD11c^{+}MHC-II^{hi}CD8^{+}DEC-205^{+}) and BTLA^{+} regulatory dendritic cells were measured with flow cytometry. Data are shown as mean ± SEM.



Online Figure XI. Female *Ldlr*$^{-/-}$ mice (n=5-6/group) were fed a WTD for 2 weeks while receiving intraperitoneal injections with PBS, an isotype antibody, an agonistic BTLA antibody, anti-CD20 or anti-CD20+BTLA. **(A)** Flow cytometry quantifications of total CD19^{+} T cells. **(B)** Flow cytometry quantifications of follicular B cells (CD21^{int}CD23^{+}), marginal zone B cells (CD21^{hi}CD23^{-}) and new B cells (CD21^{-}CD23^{-}) within the CD19^{+} population. **(C)** Flow cytometry quantifications of co-receptor expression on CD19^{+} B cells. **(D)** Flow cytometry quantification of CD4^{+} T cells and (E) of TNFα^{+} CD4^{+} T cells. Data are shown as mean ± SEM. A One-way ANOVA was performed (*p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).



Online Figure XII. **BTLA activation leads to changes in CD4 T cells.** Female *Ldlr*$^{-/-}$ mice were fed a WTD for 10 weeks after wich one group was sacrificed (baseline) and other mice were treated twice a week intraperitoneally with an isotype antibody or an agonistic BTLA antibody for 6 weeks **(A)** Flow cytometry quantifications of total CD4$^+$ T cells, naive CD4$^+$ T cells (CD62l$^+$CD44$^-$), effector CD4$^+$ T cells (CD62l$^-$CD44$^+$) and effector memory CD4 T cells (CD62l$^+$CD44$^+$) in splenocytes. **(B)** Flow cytometry quantifications of regulatory T cells (FoxP3$^+$), Th17 (RORyt$^+$), Th1 (T-bet$^+$), and Th2 (Gata-3$^+$) cells in splenocytes. (C) Data are shown as mean ± SEM. A Student's t-test was performed (*p<0.05, ** p<0.01). Tregs; regulatory T cells, Th; T helper cell.



Online Figure XIII. **Activation of BTLA promotes plaque stabilization.** Female *Ldlr*$^{-/-}$ mice were fed a WTD for 10 weeks after wich one group was sacrificed (baseline) and other mice were treated twice a week intraperitoneally with an isotype antibody or an agonistic BTLA antibody for 6 weeks. **(A)** Representative pictures and quantification of necrotic core analysis from Masson's Trichrome stained sections. **(B)** Representative pictures of MOMA-2 staining in aortic root sections with macrophage quantification. **(C)** Representative pictures of alpha-smooth muscle staining in aortic root sections with smooth muscle cell quantification. Scale bars are 100 µm. Data are shown as mean ± SEM. A One-way ANOVA was performed (*p<0.05, ** p<0.01).

Table 1

Atherosclerosis initiation study (acquired on FACS canto II)			
Treg/Th17			
Antigen	**Label**	**Clone**	**Supplier**
Dead/Live	APC-Cy7	n/a	Thermo Fisher
CD4	PerCP	RM4-5	BD Biosciences
CD25	APC	PC61.5	Thermo Fisher
CD44	FITC	IM7	Thermo Fisher
FoxP3	Pacific Blue	FJK-16s	Thermo Fisher
RORyt	PE	AFKJS-9	Thermo Fisher
Th1/Th2			
Dead/Live	APC-Cy7	n/a	Thermo Fisher
CD8	Alexa fluor 700	53-6.7	Biolegend
CD4	PerCP	RM4-5	BD Biosciences
CD62l	FITC	MEL-14	Thermo Fisher
CD44	Pe-Cy7	IM7	Thermo Fisher
T-bet	Alexa fluor 647	4B10	Thermo Fisher
Gata-3	PE	TWAJ	Thermo Fisher
B1/B2 cells			
Dead/Live	APC-Cy7	n/a	Thermo Fisher
CD19	PeCy7	1D3	Thermo Fisher
B220	FITC	RA3-6B2	Thermo Fisher
CD5	BV421	53-7.3	BD Biosciences
CD11b	APC	M1/70	Thermo Fisher
B2 cell subsets			
Dead/Live	APC-Cy7	n/a	Thermo Fisher
CD19	PeCy7	1D3	Thermo Fisher
CD21	BV421	7G6	Thermo Fisher
CD23	PE	B3B4	Thermo Fisher
CD93	PerCP-Cy 5.5	AA4.1	Thermo Fisher
IgD	APC	11-26	Thermo Fisher
IgM	FITC	II/41	Thermo Fisher
Regulatory B cells			
Dead/Live	APC-Cy7	n/a	Thermo Fisher
CD19	PeCy7	1D3	Thermo Fisher
CD5	BV421	53-7.3	BD Biosciences
CD1d	PerCP-Cy 5.5	1B1	Thermo Fisher
TIM-1	PE	RMT1-4	Biolegend
CD9	FITC	KMC8	Thermo Fisher
IL-10	APC	JES5-16E3	eBioscience

BTLA expression in human tissue			
B cell subsets			
Antigen	Label	Clone	Supplier
Dead/Live	APC-efluor 780	n/a	Thermo Fisher
CD19	PercP-Cy5.5	HIB19	Thermo Fisher
CD20	FITC	2H7	Thermo Fisher
CD27	Brilliant Violet 605	O323	Biolegend
CD45	APC	5B1	Miltenyi Biotec
BTLA	Pe-Dazzle	MIH-26	Biolegend
CD3	eFluor 450	OKT3	Thermo Fisher
IgD	Alexa fluor 700	IA6-2	Biolegend

Atherosclerosis progression study (acquired on Cytoflex S)			
B cell subsets			
Antigen	Label	Clone	Supplier
Dead/Live	APC-efluor 780	n/a	Thermo Fisher
CD93	PE-Cy7	AA4.1	Thermo Fisher
CD19	BV605	6D5	Biolegend
CD43	APC	S11	Biolegend
CD5	FITC	53-7.3	Thermo Fisher
CD21	BV421	7E9	Biolegend
CD23	PE	B3B4	Thermo Fisher
B220	PerCP-Cy5.5	RA3-6B2	Thermo Fisher
T cell subsets			
Dead/Live	APC-efluor 780	n/a	Thermo Fisher
CD4	PerCP	RM4-5	BD Biosciences
CD8a	Alexa fluor 700	53-6.7	Biolegend
CD44	BV510	IM7	Biolegend
CD62L	FITC	MEL-14	Thermo Fisher
CD25	APC	PC61.5	Thermo Fisher
FoxP3	Pacific blue	FJK-16s	Thermo Fisher
T-bet	PE-Cy7	eBio4B10	Thermo Fisher
RORyt	BV650	Q31-378	BD Biosciences
Gata-3	PE	TWAJ	Thermo Fisher
Regulatory B cells			
Dead/Live	APC-Cy7	n/a	Thermo Fisher
CD19	BV605	6D5	Biolegend
CD5	BV421	53-7.3	BD Biosciences
CD1d	PerCP-Cy 5.5	1B1	Thermo Fisher
TIM-1	PE	RMT1-4	Biolegend
IL-10	APC	JES5-16E3	eBioscience

BTLA expresion in LDLRKO mice (acquired on Cytoflex S)			
B cell subsets			
Antigen	Label	Clone	Supplier
Dead/Live	APC-efluor 780	n/a	Thermo Fisher
CD93	PE-Cy7	AA4.1	Thermo Fisher
CD19	BV605	6D5	Biolegend
BTLA	APC	8F4	Biolegend
CD5	FITC	53-7.3	Thermo Fisher
CD21	BV421	7E9	Biolegend
CD23	PE	B3B4	Thermo Fisher
B220	PerCP-Cy5.5	RA3-6B2	Thermo Fisher
T cell subsets			
Dead/Live	APC-efluor 780	n/a	Thermo Fisher
CD4	PerCP	RM4-5	BD Biosciences
CD8a	Alexa fluor 700	53-6.7	Biolegend
CD44	BV510	IM7	Biolegend
CD62L	FITC	MEL-14	Thermo Fisher
BTLA	APC	8F4	Thermo Fisher
FoxP3	Pacific blue	FJK-16s	Thermo Fisher
T-bet	PE-Cy7	eBio4B10	Thermo Fisher
RORyt	BV650	Q31-378	BD Biosciences
Gata-3	PE	TWAJ	Thermo Fisher
T cell subsets			
Dead/Live	APC-efluor 780	n/a	Thermo Fisher
CD8a	APC-efluor 700	53-6.7	Biolegend
CD19	PE-Cy7	1D3	Thermo Fisher
CD4	PerCP	RM4-5	BD Biosciences
CD11c	FITC	N418	Thermo Fisher
CD11b	BV421	M1/70	Thermo Fisher
LY-6G	PE	1A8	BD Biosciences
F4/80	Brilliant Violet 650	T45-2342	BD Biosciences
BTLA	APC	8F4	Thermo Fisher