The aging B cell landscape in atherosclerosis

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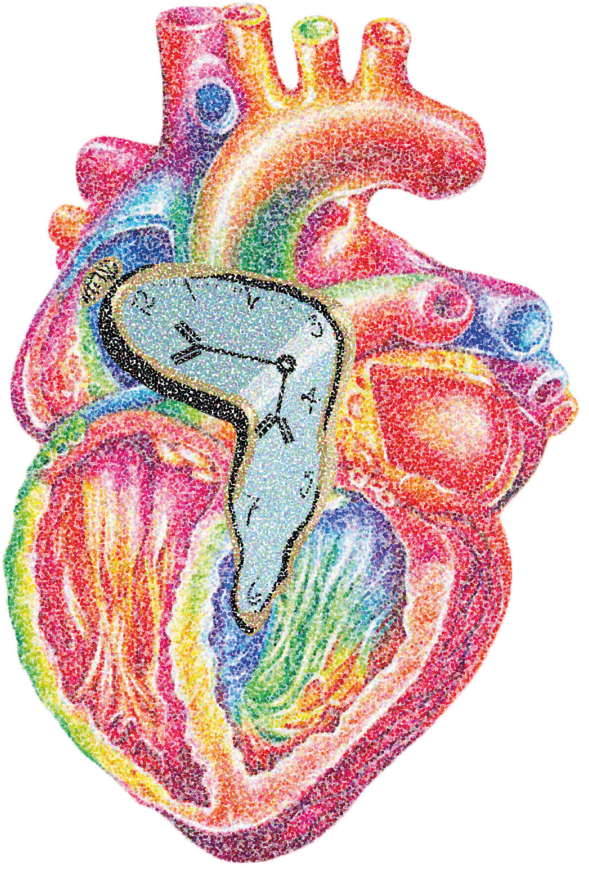

Chapter 7

IFNγ-stimulated B cells inhibit T follicular helper cells and protect against atherosclerosis

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ABSTRACT

B and T cells are interconnected in the T follicular helper – germinal center B cell (T_{FH}-GC B cell) axis, which is hyperactive during atherosclerosis development and loss of control along this axis results in exacerbated atherosclerosis. Inhibition of the T_{FH} – GC B cell axis can be achieved by providing negative co-stimulation to T_{FH} cells through the PD-1/PD-L1 pathway. Therefore, we investigated a novel therapeutic strategy using PD-L1-expressing B cells to inhibit atherosclerosis. We found that IFNγ-stimulated B cells significantly enhanced PD-L1 expression and limited T_{FH} cell development. To determine whether IFNγ-B cells can reduce collar-induced atherosclerosis, *Apoe*$^{-/-}$ mice fed a Western-type diet were treated with PBS, B cells or IFNγ-B cells for a total of 5 weeks following collar placement. IFNγ-B cells significantly increased PD-L1^{hi} GC B cells and reduced plasmablasts. Interestingly, IFNγ-B cells–treated mice show increased atheroprotective Tregs and T cell-derived IL-10. In line with these findings, we observed a significant reduction in total lesion volume in carotid arteries of IFNγ-B cells-treated mice compared to PBS-treated mice and a similar trend was observed compared to B cell-treated mice. In conclusion, our data show that IFNγ-stimulated B cells strongly upregulate PD-L1, inhibit T_{FH} cell responses and protect against atherosclerosis.

Keywords: cardiovascular disease, atherosclerosis, B cells, PD-L1, interferon-gamma, *Apoe*$^{-/-}$ mice

INTRODUCTION

Cardiovascular disease (CVD) remains a major global health problem despite great developments in diagnosis and treatment. The underlying cause for CVD is atherosclerosis, which is a chronic autoimmune-like disease and is characterized by the formation of lipid-rich lesions in the arteries. The current available treatments are aimed at lipid lowering and lead to a 25-30% relative risk reduction, indicative of an urgent need for novel disease-modifying drugs. In the last decade, accumulating evidence identified the immune system as a major contributor to the pathology of atherosclerosis.[1] For this reason, considerable effort has been devoted to restore the dysregulation of the immune system and inflammatory pathways in atherosclerosis.

B and T lymphocyte-dependent immune responses play a key role in the pathophysiology of atherosclerosis and the role of most B and T cell subsets in atherosclerosis is now well-defined. For instance, B1 cells have shown a consistent atheroprotective effect[2–5], while in multiple studies B2 cells were seen to contribute to atherosclerosis.[6–9] In addition, the involvement of Th1, Th2 and Treg cells in atherosclerosis has also been examined comprehensively as reviewed in.[10] In contrast, only recently the contribution of other leukocyte subpopulations such as follicular (FO) B cells, marginal zone (MZ) B cells and follicular helper T cells (T_{FH}) to atherosclerosis have been identified. Although FO B cells, MZ B cells and T_{FH} cells are radically different cell types, they appear to be interconnected in the T_{FH} – Germinal center B cell axis. FO B cells enter germinal centers, subsequently undergo proliferation and isotype switching and can differentiate in short-lived plasmablasts, which can further differentiate into long-lived plasma cells or memory cells. FO B cells promote the recruitment of T_{FH} cells and the generation of germinal centers through expression of inducible co-stimulator ligand.[11] Previously, it has been shown that the T_{FH} – GC B cell axis is hyperactivated and promotes lesion formation in both apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice[12] and low-density lipoprotein receptor-deficient mice fed a hypercholesterolemia-inducing diet.[13] In addition, both T_{FH} cells and FO B cells are proatherogenic and can aggravate atherosclerosis.[14,15]

The T_{FH} – GC B cell axis can be regulated by the co-inhibitory programmed death-1 (PD-1)/PD-L1 pathway. T_{FH} cells highly express PD-1 and their accumulation can be controlled by MZ B cells which express PD-L1. In response to high cholesterol levels, MZ B cells upregulate the expression of PD-L1 and thereby regulate T_{FH} cell accumulation which limits an exacerbated adaptive immune response.[13] However, this mechanism fails to completely arrest disease development. Interestingly, in autoimmune encephalomyelitis, it has been shown that adoptive transfer of B cells expressing high levels of PD-L1 limited disease severity.[16] Whether PD-L1-expressing B cells can also be used therapeutically to inhibit atherosclerosis development has not yet been reported. In this study, we therefore induced PD-L1-expressing B cells and investigated whether adoptive transfer of these cells could inhibit atherosclerosis development.

MATERIALS AND METHODS

Animals

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. Female C57BL/6, B6.SJL-PtprcaPepcb/BoyCrl (also known as CD45.1) and apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice were bred in house and kept under standard laboratory conditions. Diet and water were provided *ad libitum*. All injections were administered i.v. to the lateral tail vein 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 (0.1 mg/ml), and xylazine (8 mg/ml). Mice were bled and perfused with phosphate-buffered saline (PBS) through the left cardiac ventricle.

Cell culture

B cells were isolated from splenocytes of C57BL/6 or *Apoe*$^{-/-}$ mice using CD19^{+} microbeads (Miltenyi Biotec) and cultured in complete RPMI medium. Isolated B cells were cultured with different concentrations of heat killed *Staphylococcus aureus* (Invivogen), B-cell activating factor (BAFF; R&D systems) or interferon-gamma (IFNγ; ThermoFisher) for 24 hours. For co-culture experiments, CD4^{+} T cells were isolated from splenocytes of C57BL/6 mice using a CD4^{+} T cell isolation kit (Miltenyi Biotec). For some experiments, the CD4^{+} T cells were labeled using a CellTrace Violet Cell Proliferation kit (ThermoFisher) according to the instructions of the manufacturer. B and T cells were co-cultured and stimulated with an agonistic plate-bound CD3 antibody (5 µg/ml) for 72 hours. For adoptive transfer experiments, B cells were isolated from splenocytes of *Apoe*$^{-/-}$ or CD45.1 mice, cultured in RPMI medium and stimulated for 24 hours with 20 ng/ml of IFNγ. Subsequently, cells were washed, checked for purity using flow cytometry and resuspended for injections with PBS. For the injection of untouched B cells, B cells were freshly isolated from splenocytes of *Apoe*$^{-/-}$ or CD45.1 mice and directly used for adoptive transfer experiments.

Real-time quantitative PCR

RNA was extracted from cultured B cells by using Trizol reagent according to manufacturer's instructions (Invitrogen) after which cDNA was generated using RevertAid M-MuLV reverse transcriptase according to the instructions of the manufacturer (Thermo Scientific). Quantitative gene expression analysis was performed using Power SYBR Green Master Mix on a 7500 Fast Real-Time PCR system (Applied Biosystems). Gene expression was normalized to housekeeping genes. Primer sequences are available in **Supplementary table 1**.

***In vivo* experiments**

For all *in vivo* experiments, *Apoe*$^{-/-}$ mice were used. *Apoe*$^{-/-}$ mice were fed a Western-type diet (WTD) containing 0.25% cholesterol and 15% cacao butter (SDS, Sussex, UK). For the pilot study, mice were pre-fed a WTD for 2 weeks and were subsequently treated with 2x10^{6} freshly isolated or cultured B cells and sacrificed after three days. To assess the impact of WTD on the percentage of PD-L1^{hi} B cells,

apoE$^{-/-}$ mice were fed a chow diet or a WTD for 2 or 7 weeks. For atherosclerosis experiments, carotid artery plaque formation was induced after two weeks of WTD feeding by perivascular collar placement in these mice as described previously.[17] Mice continued to be fed a WTD for 5 weeks and during this period received 3 injections with either freshly isolated B cells ($2x10^6$ cells/injection), B cells stimulated with 20 ng/ml IFNγ for 24 hours ($2x10^6$ cells/injection) or PBS. To discriminate between adoptively transferred cells and endogenous cells, B cells were isolated from CD45.1 mice for the last injection. Time between injections was 2 weeks. At the end of experiments, mice were sacrificed and relevant organs were harvested for analysis.

Cytokine analysis

Isolated splenocytes from PBS, B cell or IFNγ-B cell treated mice were cultured in complete RPMI medium and stimulated for 72 hours with anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml). The levels of cytokines 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.

Flow cytometry

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. Next, cells were stained with anti-mouse fluorochrome-conjugated antibodies (**Supplementary table 2**). Regular flow cytometry was performed on a Cytoflex S (Beckman Coulter) and the acquired data were analyzed using FlowJo software. Gates were set according to isotype or fluorescence minus one controls.

Serum measurements

Serum was acquired by centrifugation and stored at -20°C until further use. Total serum titers of IgM, IgG1, IgG2c and oxidized LDL-specific antibodies were quantified by ELISA as previously described.

Histology

Carotid arteries and hearts were frozen in OCT compound (TissueTek) and stored at -80°C until further use. Transverse cryosections proximal to the collar were collected and mounted on Superfrost adhesion slides (ThermoFisher). To determine lesion size, cryosections were stained with hematoxylin and eosin (Sigma-Aldrich). Quantification of lesion size was assessed every 100 μm from the first section with visible lesion proximal to the collar until no lesion could be observed. Plaque volume was determined with lesion size and the total distance of the lesions in the carotid artery. Phenotypic analysis of the lesion was performed on sections containing the largest three lesions. Collagen content in the lesion was assessed with a Masson's trichrome staining according to the manufacturers protocol (Sigma-Aldrich). Necrotic core size was determined manually by selecting acellular areas in the Masson's trichrome stained sections and shown as absolute area and percentage of the total plaque area. 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 using the ImmPACT NovaRED substrate (Vector Laboratories). For the detection of vascular smooth muscle cells, cryosections were stained with a monoclonal rat alpha-smooth muscle actin antibody conjugated to Alexa fluor 647 (1:1500, Novus Biologicals). Furthermore, cryosections were stained with a monoclonal rat CD4 antibody conjugated to FITC (1:150, eBioscience) and a monoclonal rat IgG2b isotype control conjugated to FITC (1:150, MBL) to determine $CD4^+$ T cell infiltration. For all fluorescent images, cryosections were blocked with αCD16/32 Fc block (1:250, Biolegend) and nuclei were stained with DAPI. All slides were analyzed with a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems).

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 Holm-Sidak post hoc test. Probability values of $p < 0.05$ were considered significant. All statistical analyses were performed using GraphPad Prism.

RESULTS

IFNγ-stimulated B cells express high levels of PD-L1

Previously, it has been shown that hypercholesterolemia promotes PD-L1 expression on B cells in *Ldlr*$^{-/-}$ mice, which can regulate T_{FH} cell accumulation, limiting an exacerbated adaptive immune response.[13] In order to investigate if the administration of a Western-type diet (WTD) also affects PD-L1-expressing B cells in *Apoe*$^{-/-}$ mice, we compared PD-L1 expression on B cells from chow fed *Apoe*$^{-/-}$ mice and *Apoe*$^{-/-}$ mice fed a WTD for 2 and 7 weeks using flow cytometry (**Fig. 1A** and **Fig. S1A**). Whereas we observed no significant differences in PD-L1^{hi} B cells between chow fed *Apoe*$^{-/-}$ mice and *Apoe*$^{-/-}$ mice fed a WTD for 2 weeks, longer administration of WTD increases the percentage of PD-L1^{hi} B cells, in line with previous findings. Nonetheless, this elevation in PD-L1 expressing B cells upon hypercholesterolemia is not sufficient to halt disease development[13], indicating the need to further stimulate the regulation of the T_{FH} – GC B cell axis through the PD-1/PD-L1 pathway. We therefore aimed to generate a population of PD-L1^{hi} B cells *ex vivo*, to adoptively transfer in WTD fed *Apoe*$^{-/-}$ mice to halt atherosclerosis. We explored several stimuli that previously have been shown to control PD-L1 expression[18,19] (**Fig. S1B-C** and **Fig. 1**) and found that IFNγ dose-dependently increased the number of PD-L1$^+$ B cells, with 20.0 ng/ml of IFNγ leading to an almost pure population of PD-L1-expressing B cells (**Fig. 1B**). Using qPCR, we also found an almost 8-fold induction of PD-L1 on mRNA level after IFNγ stimulation (**Fig. 1C**). Furthermore, we observed that the majority of IFNγ-stimulated B cells expressed very high levels of PD-L1 (**Fig. 1D**). Previously, it has been shown that IFNγ-signaling in B cells drives STAT-1 dependent expression of T-bet and BCL-6 and switches them towards a GC B cell phenotype with increased IFNγ and IL-6 production.[20,21] In line with this, our *ex vivo* IFNγ-stimulated B cells (IFNγ-B cells) showed a similar increase in STAT1, T-bet and BCL-6 (**Fig. 1E**) gene expression. In contrast, we observed a trend towards less IL-6 and no difference in IFNγ expression (**Fig. S1D**). Interestingly, IFNγ stimulation resulted in a strong significant increase in TGFβ expression (**Fig. 1E**). We also investigated the chemokine receptor profile of these B cells and found a dose-dependent increase in CCR7 expression (**Fig. 1E**), while there was no effect on CXCR5 or Ebi-2 (**Fig. S1D**). This change in

chemokine receptor expression is typical of B cells that migrate towards the T-B cell border in lymphoid tissues in response to the CCL21 gradient.[22] Hence, these data indicate that IFNγ-stimulated B cells express high levels of PD-L1 and TGFβ and a chemokine profile that homes B cells to the T-B cell border. In line with these findings, we observed that IFNγ stimulation of B cells not only induced coinhibitory PD-L1 expression on all B cells (**Fig. 1C**) but also increased the percentage of GC B cells and MZ B cells (**Fig. 1F**). Together with a decrease in FO B cells, we thus show that IFNγ stimulation generates a B cell population with an enhanced anti-inflammatory phenotype.

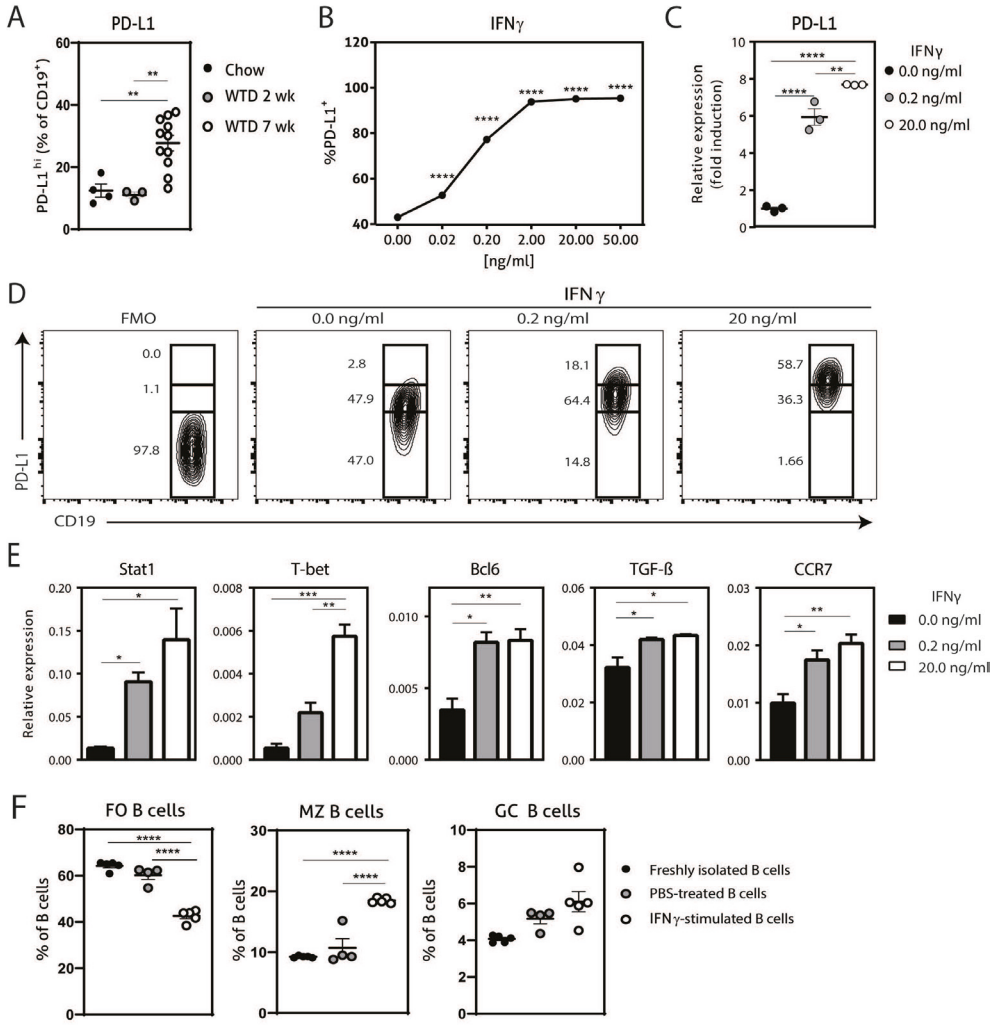

Figure 1. Characterization of IFNγ-stimulated B cells. (A) PD-L1^{hi} expressing CD19^{+} B cells were determined in spleens of *Apoe*$^{-/-}$ mice fed a regular Chow diet or Western type diet for 2 or 7 weeks using flow cytometry. **(B)** CD19^{+} B cells were isolated from C57BL/6 mice and stimulated for 24 hours with different doses interferon-gamma (IFNγ) after which PD-L1 protein expression was measured with flow cytometry. **(C)** CD19^{+} B cells were unstimulated or stimulated with 0.2 ng/ml or 20.0 ng/ml IFNγ for 24 hours, after which mRNA expression of PD-L1 was assessed using qPCR. **(D)** B cells as stimulated in **(C)** were analyzed for PD-L1^{lo}, PD-L1^{int} and PD-L1^{hi} expression with flow cytometry. **(E)** mRNA expression was analyzed for depicted genes in B cells as stimulated in **(C)**. **(F)** FO B cells (CD23^{+}) MZ B cells (CD23^{-} CD21^{+}) and GC B cells (GL-7^{+} CD95^{+}) were determined in CD19^{+} B cells stimulated with 20.0 ng/ml IFNγ for 24 hours. Data are analyzed with a One-Way ANOVA and shown as mean ± SEM (* p<0.05, ** p<0.01, ***p<0.001, ****p<.00001). n=3-11/group.

IFNγ-stimulated B cells inhibit T_{FH} cells *in vitro* and *in vivo*

Next, we explored the functional effects of IFNγ-stimulated B cells on T_{FH} cell development using a $CD4^+$ T cell and B cell coculture. We stimulated wildtype $CD4^+$ T cells for 72 hours with anti-CD3 in the presence of unstimulated or IFNγ-stimulated *Apoe*$^{-/-}$ B cells. Although we did not observe any difference in proliferative capacity of $CD4^+$ T cells (**Fig. 2A**), we found a remarkable decrease of T_{FH} cells when $CD4^+$ T cells were cocultured with IFNγ-stimulated B cells compared to unstimulated B cells (**Fig 2B**). These findings illustrate that IFNγ-B cells are able to curb T_{FH} cell development *in vitro*. We subsequently tested IFNγ-stimulated B cells in an *in vivo* setting by adoptively transferring either freshly isolated B cells or B cells stimulated with 20.0 ng/ml of IFNγ for 24 hours into *Apoe*$^{-/-}$ mice. To induce initial T_{FH} cell accumulation, mice were fed a Western-type diet for two weeks before they received the adoptive transfers (**Fig. 2C**).[13] We observed a remarkable reduction in effector $CD4^+$ T cells in mice treated with IFNγ-B cells compared to mice that received PBS or B cells (**Fig. 2D**). Contrary, the number of naïve $CD4^+$ T cells was increased in IFNγ-B cells treated mice compared to mice receiving PBS (**Fig. 2D**). Most importantly, treatment of mice with IFNγ-B cells resulted in a strong reduction in T_{FH} cells compared to mice that were administered with PBS or B cells (**Fig. 2E**). These data demonstrate that IFNγ-B cells are also able to inhibit T_{FH} cells *in vivo*.

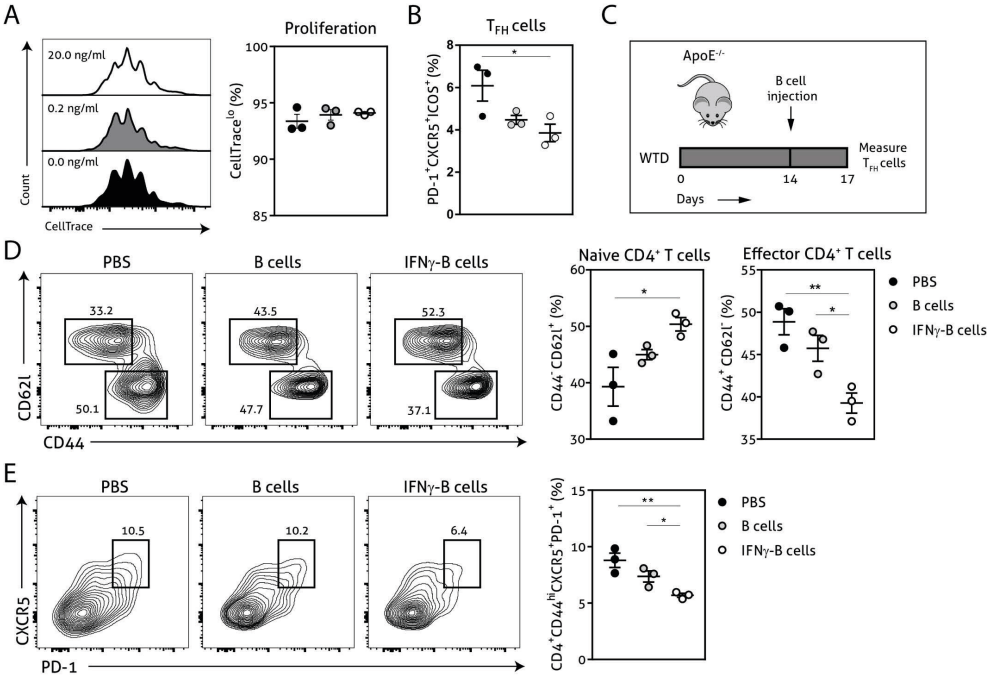

Figure 2. IFNγ-B cells inhibit T follicular helper cells *in vitro* and *in vivo*. Isolated $CD19^+$ B cells were unstimulated or stimulated with 0.2 ng/ml or 20.0 ng/ml IFNγ for 24 hours and co-cultured with isolated $CD4^+$ T cells. The coculture was stimulated with anti-CD3 (5 μg/ml) for 72 hours after which **(A)** proliferation of $CD4^+$ T cells was analyzed with CellTrace Violet and **(B)** the number of follicular $CD4^+$ T helper (T_{FH}) cells were analyzed. **(C)** *Apoe*$^{-/-}$ mice were fed a Western type diet for 2 weeks after which they either received PBS, freshly isolated $CD19^+$ B cells (B cells) or $CD19^+$ B cells stimulated with 20.0 ng/ml IFNγ for 24 hours (IFNγ-B cells). After three days, mice were sacrificed and spleens were isolated for flow cytometry analysis for **(D)** naive ($CD62l^+CD44^-$) and effector ($CD62l^-CD44^{hi}$) $CD4^+$ T cells and **(E)** T follicular helper cells ($CD4^+CD44^{hi}CXCR5^+PD\text{-}1^+$). Representative flow charts of the $CD4^+$ T cell population are shown. Data are analyzed with a One-Way ANOVA and shown as mean ± SEM (* $p<0.05$, ** $p<0.01$). n=3/group.

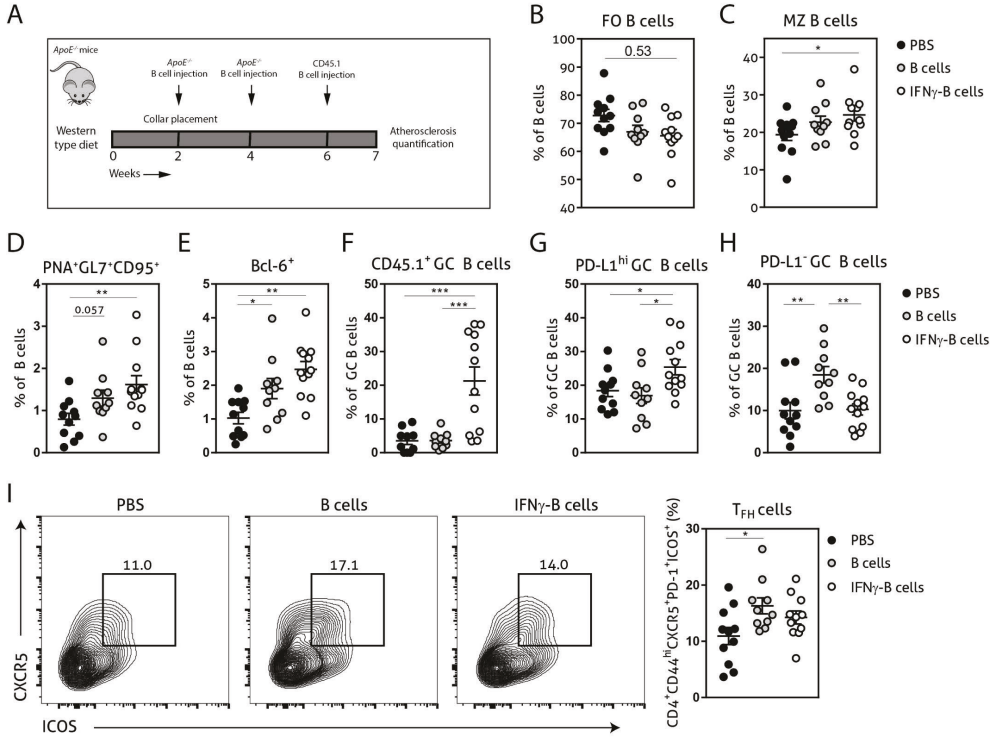

Figure 3. Adoptive transfer of IFNγ-B cells during atherosclerosis development affects the T_{FH} – GC B cell axis. (A) *Apoe*$^{-/-}$ mice were fed a Western type diet for 7 weeks. After 2 weeks they received a perivascular collar and were treated with PBS, freshly isolated B cells (B cells) or B cells stimulated with 20.0 ng/ml IFNγ for 24 hours (IFNγ-B cells). Mice received a total of three injections and injections were spaced every two weeks. After 7 weeks, mice were sacrificed and spleens were analyzed with flow cytometry. Flow cytometry quantification of **(B)** follicular (FO) B cells, **(C)** marginal zone (MZ) B cells, (D) extracellular staining of germinal center B cells, **(E)** intracellular staining of germinal center B cells, **(F)** CD45.1^{+} germinal center B cells, **(G)** PD-L1^{hi} germinal center B cells, **(H)** PD-L1^{-} germinal center B cells and **(I)** flow charts and quantification of follicular helper T cells (CD44^{hi}PD-1^{+}CXCR5^{+}ICOS^{+}). Data are analyzed with a One-Way ANOVA and shown as mean ± SEM (* p<0.05, ** p<0.01, *** p<0.001). n=10-12/group.

Adoptive transfer of IFNγ-B cells during atherosclerosis development affects the T_{FH} – GC B cell axis

Given the *in vitro* and *in vivo* regulatory effects of IFNγ-B cells on T_{FH} cells, we further investigated whether these B cells would be able to restrict T_{FH} cell numbers during atherosclerosis development. We fed *Apoe*$^{-/-}$ mice a Western-type diet for 2 weeks after which we placed a perivascular collar and started treatment with either PBS, B cells or IFNγ-B cells (**Fig. 3A**). After a total of 3 injections and 7 weeks of Western-type diet, we harvested the organs and analyzed the immune cells associated with the T_{FH} – GC B cell axis. IFNγ-B cell-treated mice showed a trend towards reduced FO B cells (**Fig. 3B**) and a significant increase in MZ B cells compared to mice receiving PBS (**Fig. 3C**). Moreover, mice that received IFNγ-B cells showed a significant increase in the number of GC B cells compared to mice that were administered PBS (**Fig. 3D** and **E**). By using the CD45 congenic marker system, we showed that adoptively transferred IFNγ-B cells indeed reached the germinal center (**Fig. 3F**) and locally increased the number of PD-L1^{hi} B cells (**Fig. 3G**). As shown in **Fig. 3D** and **E**, freshly isolated

B cells also increased the number of GC B cells, but these were not derived from the adoptive transfer (**Fig. 3F**) and were PD-L1^{-} (**Fig. 3H**). At sacrifice, we did not observe a difference in T_{FH} cells in IFNγ-B cell-treated mice, while adoptive transfer of B cells resulted in an increase in T_{FH} cells compared to PBS-treated mice (**Fig. 3I**).

Next, we assessed the number of plasmablasts and plasma cells and found a significant reduction in plasmablasts when mice received IFNγ-B cells compared to mice receiving PBS (**Fig. 4A**). Mice that received B cells showed a similar trend towards less plasmablasts and also a significant increase in plasma cells compared to PBS- and IFNγ-B cells-treated mice. Since BLIMP-1 is the driving transcription factor for plasma cell generation[23], we also measured BLIMP-1 expression which revealed a significant increase in BLIMP-1^{+} cells in mice that received B cells compared to mice receiving PBS or IFNγ-B cells (**Fig. 4B**). Since plasmablasts and plasma cells are responsible for the humoral immunity, we measured circulating antibodies. However, neither B cell treatments led to a significant difference in circulating antibodies (**Fig. 4C**).

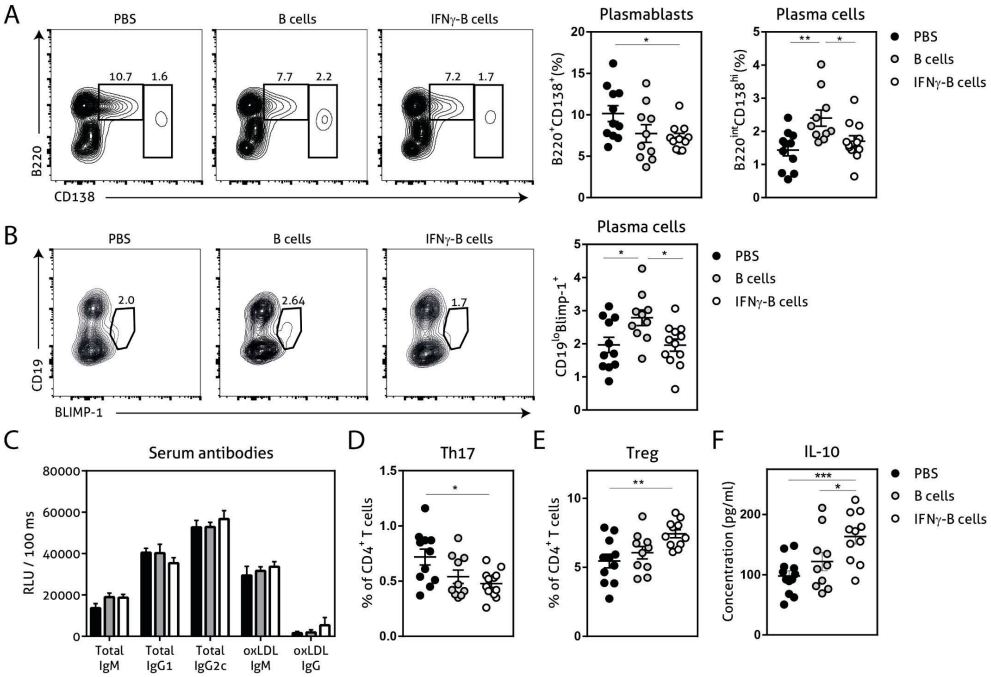

Figure 4. Effects of adoptive transfer of IFNγ-B cells on the humoral immunity. *Apoe*$^{-/-}$ mice were fed a Western type diet for 7 weeks. After 2 weeks they received a perivascular collar and were treated with PBS, freshly isolated B cells (B cells) or B cells stimulated with 20.0 ng/ml IFNγ for 24 hours (IFNγ-B cells). Mice received a total of three injections and injections were spaced every two weeks. After 7 weeks, mice were sacrificed and spleens were analyzed with flow cytometry. **(A)** Flow charts and quantification of plasmablasts (B220^{+}CD138^{+}) and plasma cells (B220^{lo}CD138^{hi}). **(B)** Flow charts and quantification of CD19^{lo}BLIMP-1^{+} plasma cells. **(C)** Serum was analyzed for circulating antibodies by ELISA. Spleens were analyzed with flow cytometry for **(D)** Th17 cells (RORγt^{+}) or **(E)** Treg cells (FoxP3^{+}). **(F)** Splenocytes were isolated and stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml) for 72 hours after which supernatant was collected and analyzed for cytokine expression with a multiplex analysis. Quantification of IL-10 concentration is shown. Data are analyzed with a One-Way ANOVA and shown as mean ± SEM (* $p<0.05$, ** $p<0.01$). n=10-12/group.

Adoptive transfer of IFNγ-B cells promotes an anti-inflammatory CD4^{+} T cell response

The CD4^{+} T cell response is also highly involved in the pathogenesis of atherosclerosis and PD-L1^{hi}

B cells have previously shown to restrict CD4$^+$ T cell differentiation.[16] While we did not observe any differences in Th1 or Th2 CD4$^+$ T cells between mice treated with IFNγ-B cells, B cells or PBS in our study (**Fig. S2**), we observed a significant decrease in Th17 cells (**Fig. 4D**) and a significant increase in atheroprotective regulatory T cells (**Fig. 4E**; Tregs) in mice treated with IFNγ-B cells. We next measured cytokine levels of *ex vivo* anti-CD3 and anti-CD28 stimulated splenocytes for 72 hours with a multiplex assay. In line with the increase in Tregs, we observed a significant increase in IL-10 production by splenocytes from IFNγ-B cells treated mice compared to splenocytes from mice treated with PBS or B cells (**Fig. 4F**).

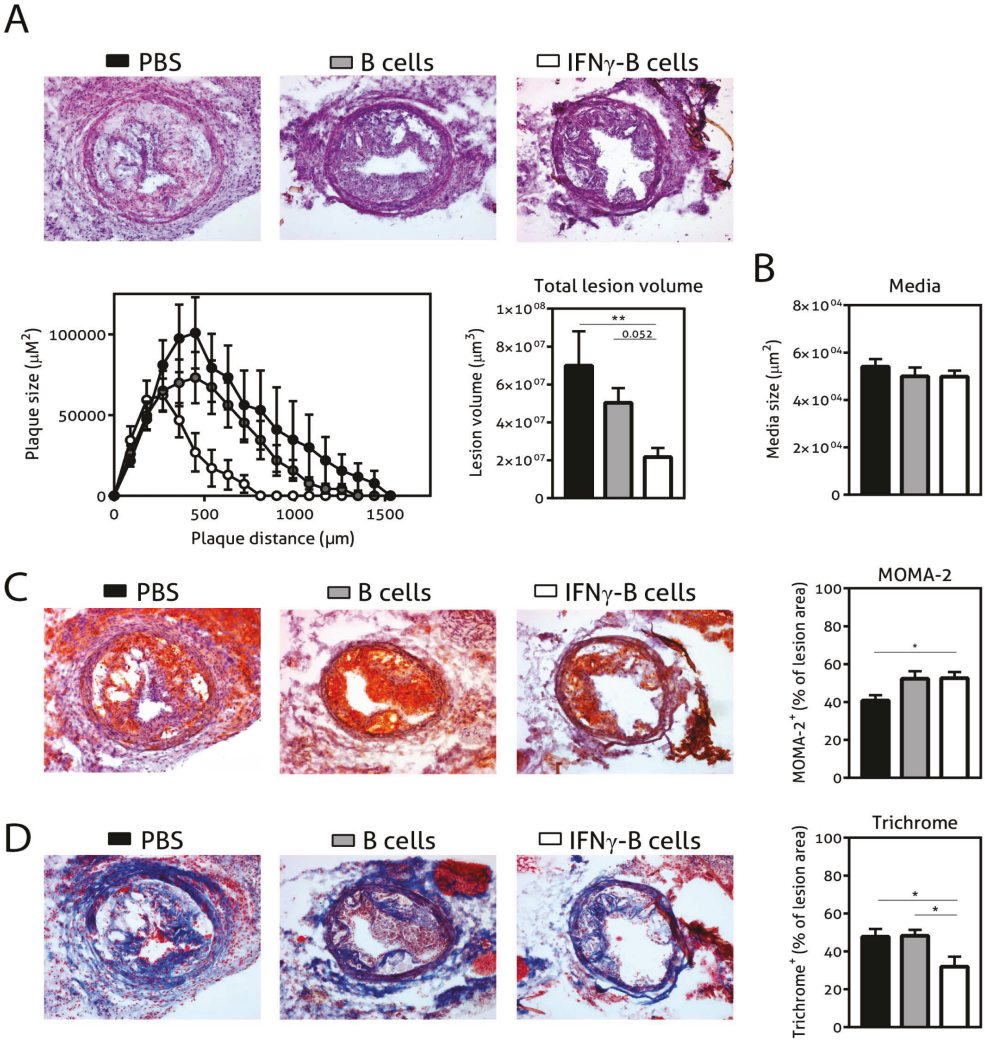

Figure 5. Adoptive transfer of IFNγ-B protects against atherosclerosis. *Apoe*$^{-/-}$ mice were fed a Western type diet for 7 weeks. After 2 weeks they received a perivascular collar and were treated with PBS, freshly isolated B cells (B cells) or B cells stimulated with 20.0 ng/ml IFNγ for 24 hours (IFNγ-B cells). Mice received a total of three injections and injections were spaced every two weeks. After 7 weeks, mice were sacrificed and **(A)** total lesion volume and **(B)** media size was determined in the right carotid artery with a hematoxylin and eosin staining. Lesion phenotype was determined in sections containing the largest lesions. **(C)** Macrophage content was analyzed using a MOMA-2 staining. **(D)** Collagen content was quantified with a Trichrome staining. Data are analyzed with a One-Way ANOVA and shown as mean ± SEM (* $p<0.05$, ** $p<0.01$). n=8-12/group.

Adoptive transfer of IFNγ-B protects against lesion formation

Given the immune-regulating effects of IFNγ-B cells, we subsequently assessed whether adoptive transfer of these IFNγ-B cells was able to reduce collar-induced atherosclerosis in *Apoe*$^{-/-}$ mice. We quantified lesion development in the carotid arteries and found a significant reduction in total lesion volume when mice were treated with IFNγ-B cells compared to PBS and a similar trend was found when compared to B cell-treated mice (**Fig. 5A**). Media size did not differ between the treatment groups (**Fig. 5B**). Furthermore, there were no differences in weight gain and serum cholesterol levels (**Fig. S3A** and **S3B**). We also assessed lesion phenotype at the site of the maximal lesion. This revealed that mice treated with IFNγ-B cells showed an early lesion phenotype with relatively more macrophages than collagen compared to mice that received B cells or PBS (**Fig. 5C** and **5D**), suggesting that adoptive transfer of IFNγ-B cells inhibited atherosclerotic lesion progression towards more advanced lesions. Evaluation of vascular smooth muscle cells (VSMCs) using α-smooth muscle actin did not reveal any differences (**Fig. S4A**) and we also did not observe altered necrotic core formation within the atherosclerotic plaque in IFNγ-B cell treated mice compared to the other groups (**Fig. S4B**). Finally, we did not observe significant differences in CD4^{+} T cell infiltration between the treatment groups (**Fig. S4C**) and only very low numbers of CD4^{+} T cells were found, suggesting that the reduction in lesion size is caused by systemic anti-atherogenic effects of PD-L1^{hi} B cells, rather than a local effect.

DISCUSSION

A proatherogenic role has been reported for the T_{FH} – GC B cell axis.[12–14] Direct depletion of T_{FH} cells in *Ldlr*$^{-/-}$ mice resulted in a reduction of atherosclerosis[14], and loss of control on the T_{FH} – GC B axis by depletion of MZ B cells[13] or CD8^{+} regulatory T cells aggravated atherosclerosis.[12] We now show that adoptive transfer of PD-L1 expressing B cells inhibit T_{FH} cell responses both *in vitro* and *in vivo* and protect against atherosclerosis.

Using *ex vivo* stimulation of B cells with IFNγ we rapidly provided a large and almost pure population of PD-L1-expressing B cells. IFNγ is an extensively investigated cytokine with a broad range of immune actions. Interestingly, while we show here that IFNγ stimulation of B cells results in a regulatory phenotype that reduces the number of T_{FH} cells, earlier work identified a role for IFNγ in the generation of spontaneous germinal centers and B cell autoreactivity.[20,24] These previous data were, however, mainly acquired in the context of autoimmunity, where B cells receive a multitude of different signals and IFNγ signaling seems primarily to synergize with BCR-, CD40- and TLR-mediated stimuli to induce spontaneous germinal centers.[24] Under these circumstances, IFNγ signaling in B cells results in increased IL-6 and IFNγ production which drives auto-immunity.[20,21] In contrast, our *ex vivo* stimulated B cells lacked additional stimuli and upregulated expression of PD-L1 and TGFβ, while we did not see any effects on IL-6 and IFNγ. We further showed that IFNγ-B cells express high levels of CCR7 with minimal changes in CXCR5 and Ebi-2. This expression profile is in line with a previous study that demonstrated that MZ B cells from *Ldlr*$^{-/-}$ mice interact with pre-T_{FH} cells at the T-B cell border in response to a high-cholesterol diet.[13] Moreover, we show that *ex vivo* IFNγ stimulation generates a PD-L1^{hi} B cell pool containing enhanced MZ and GC B cells, while pro-atherogenic FO B cells are decreased. During our atherosclerosis study, we indeed found that adoptively transferred IFNγ-B cells showed the characteristics of B cells that reside in or near the germinal center.

Similar to flow-sorted PD-L1^{hi} B cells[16], we next showed that IFNγ-B cells were able to inhibit T_{FH} cell numbers *in vitro* and in our short *in vivo* experiment. Notably, at the point of sacrifice in our atherosclerosis experiment we did not observe restriction of T_{FH} cells by IFNγ-B cells, but we did demonstrate that IFNγ-B cells were able to promote anti-inflammatory CD4$^+$ T cells. This corresponds with the effects found in experimental autoimmune encephalomyelitis using PD-L1^{hi} B cells, which restricted Th1 and Th17 differentiation.[16] Along this line, we found that IFNγ-B cells resulted in decreased Th17 cells and a significant increase in atheroprotective Tregs and IL-10 production of CD4$^+$ T cells. Interestingly, it has been reported that during atherosclerosis development there is a plasticity between Treg cells and T_{FH} cells and disturbances of this delicate balance greatly affected atherosclerosis development.[14] We did not directly investigate this plasticity in our study, but our work shows that IFNγ-B cells are able to inhibit T_{FH} cells and increase Treg cells.

Furthermore, we demonstrated atheroprotective effects of IFNγ-B cells that express high levels of PD-L1.The observed reduction in atherosclerosis upon adoptive transfer of IFNγ-B cells was accompanied by changes in plaque morphology (relatively more macrophages, reduced collagen), indicating a more initial plaque phenotype. Reduced collagen content did not coincide with alterations in overall VSMCs content. Although we cannot specifically determine collagen production rate by SMCs locally in the plaque, the lack of difference in SMC content suggests that the difference in collagen content that we observed in the plaque is not explained by the amount of SMCs. Although we did not investigate this in our study, the reduced collagen content in IFNγ-B cell treated mice may also be attributed to an increase in collagen degradation through the production of matrix metalloproteinases by the macrophages in the plaque, which were relatively increased upon IFNγ-B cell transfer. The atheroprotective effect of PD-L1^{hi} B cells is in line with a general protective role of the PD-L1/PD-1 axis in atherosclerosis. Mice deficient in both PD-L1 and PD-L2 show increased atherosclerosis.[25] Similarly, PD-1 knockout mice or mice treated with a PD-1 blocking antibody developed exacerbated atherosclerosis[26,27], whereas stimulation of PD-1 signaling reduces atherosclerosis.[28] Our data is further supported by previous studies that demonstrated the proatherogenic role of T_{FH} cells.[12–14]

However, due to the pleiotropic nature of IFNγ, other factors besides PD-L1 could also have contributed to our findings. Indeed, the observed increase in TGFβ expression could have contributed to both the T_{FH} inhibition and Treg induction, since TGFβ signaling is known to prevent T_{FH} cell accumulation and can promote Tregs.[29] In addition, there have been a large number of studies with adoptive transfer of B cells expressing TGF-β that reported immune tolerance in autoimmune mouse models.[30–33] The majority of these studies reported Treg induction, which is in line with our findings of increased Tregs and IL-10 production after adoptive transfer of IFNγ-B cells. PD-L1 is also known to be essential for the induction of Treg cells[34], the Treg accumulation could thus be a combined effect of increased TGFβ and PD-L1 expression by IFNγ-B cells. Since the atheroprotective effects of Treg cells is well characterized[10,35], the observed Treg induction undoubtedly contributed to the reduced atherosclerosis found with adoptive transfer of IFNγ-B cells. Moreover, we show that *ex vivo* IFNγ stimulation generates a pool of PD-L1^{hi} B cells which contains reduced FO B cells. FO B cells can contribute to atherosclerosis progression[15], and despite the increased co-inhibitory PD-L1 expression following IFNγ exposure, we cannot exclude that a decrease in FO B cells in the adoptively transferred IFNγ-stimulated B cells also contributed to

the observed anti-atherogenic effect.

In conclusion, this study uncovers a new role for *ex vivo* stimulation of B cells with IFNγ for the induction of atheroprotective B cells. IFNγ-B cells show the genetic makeup of GC B cells with increased expression of PD-L1 and TGFβ and effectively inhibit T_{FH} cells *in vitro* and *in vivo* and ameliorate atherosclerosis development in *Apoe*$^{-/-}$ mice. These results further emphasize the proatherogenic role of the T_{FH} – GC B axis and provide a novel way to regulate this axis.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

HD, JdM, GvP, JK and ACF contributed to the conception and design of the study; HD, JdM, JA, FS, BS, MGK, MJK, IB, GvP and ACF carried out the experiments and acquired the data; HD and JdM performed the data analysis; HD, JdM and ACF wrote the manuscript; CJB, JK and ACF provided critical feedback to the manuscript; JK and ACF supervised the project. All authors read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

Supplementary Figures and Tables are available online.

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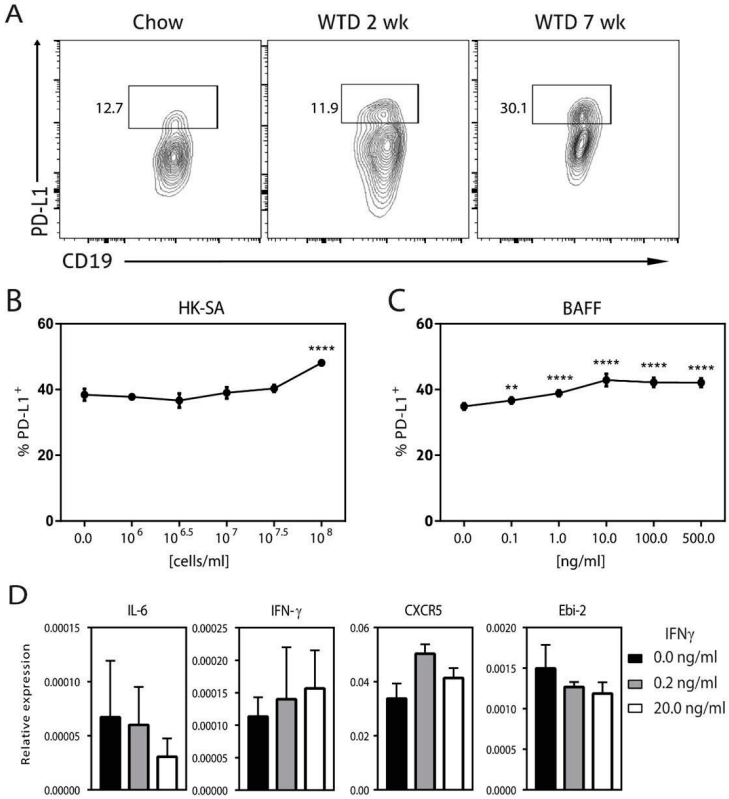
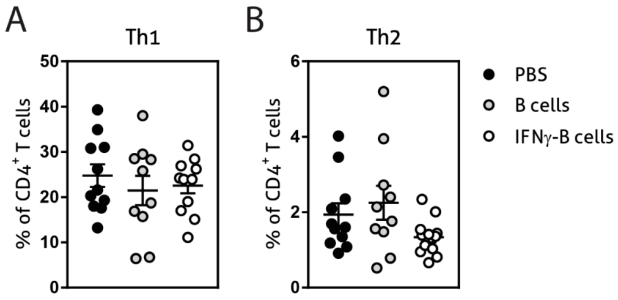
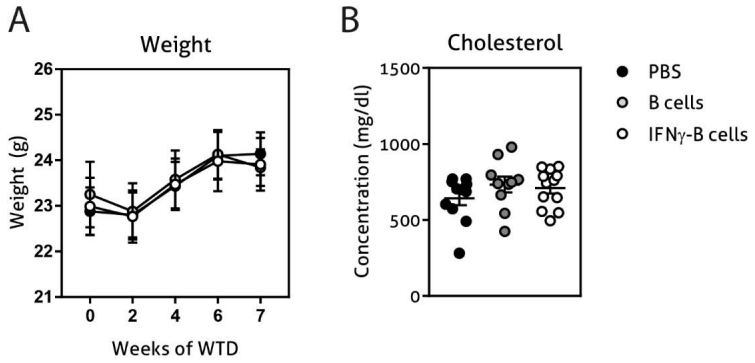
SUPPLEMENTARY FIGURES AND TABLES



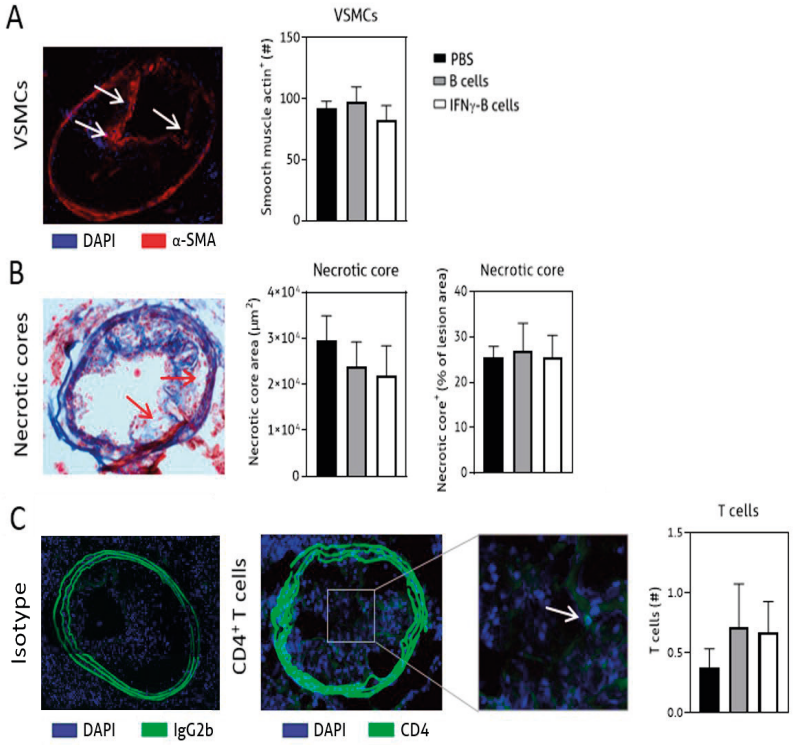
Supplementary Figure 1. PD-L1 expression on B cells. (A) Representative flow cytometry contour plots of PD-L1^{hi} expressing CD19^{+} B cells in *Apoe*$^{-/-}$ mice fed a chow diet or a WTD for 2 or 7 weeks. CD19^{+} B cells were isolated from C57BL/6 mice and stimulated for 24 hours with different doses of **(B)** heat killed *S. Aureus* (HK-SA) or **(C)** B cell activating factor (BAFF) after which PD-L1 expression was measured with flow cytometry. **(D)** CD19^{+} B cells were unstimulated or stimulated with 0.2 ng/ml or 20.0 ng/ml IFNγ for 24 hours, after which mRNA expression of depicted genes was assessed using qPCR. Data are analyzed with a One-Way ANOVA and shown as mean ± SEM (** p<0.01, ****p<.00001). n=3/group.



Supplementary Figure 2. Effects of adoptive transfer of IFNγ-B cells on Th1 and Th2 cells. *Apoe*$^{-/-}$ mice were fed a Western type diet for 7 weeks. After 2 weeks they received a perivascular collar and were treated with PBS, freshly isolated B cells (B cells) or B cells stimulated with 20.0 ng/ml IFNγ for 24 hours (IFNγ-B cells). Mice received a total of three injections and injections were spaced every two weeks. After 7 weeks, mice were sacrificed and spleens were analyzed with flow cytometry for **(A)** Th1 cells (T-bet^{+}) and **(B)** Th2 cells (Gata-3^{+}). Data are shown as mean ± SEM. n=10-12/group.



Supplementary Figure 3. Weight and cholesterol levels. *Apoe*$^{-/-}$ mice were fed a Western type diet for 7 weeks. After 2 weeks they received a perivascular collar and were treated with PBS, freshly isolated B cells (B cells) or B cells stimulated with 20.0 ng/ml IFNγ for 24 hours (IFNγ-B cells). Mice received a total of three injections and injections were spaced every two weeks. **(A)** Weight was assessed during the experiment and **(B)** and cholesterol serum levels were analyzed at the end of the experiment. Data are shown as mean ± SEM. n=10-12/group.



Supplementary Figure 4. Adoptive transfer of IFNγ-B does not affect VSMCs, necrotic core size and T cell infiltration in atherosclerotic plaques. *Apoe*$^{-/-}$ mice were fed a Western type diet for 7 weeks. After 2 weeks they received a perivascular collar and were treated with PBS, freshly isolated B cells (B cells) or B cells stimulated with 20.0 ng/ml IFNγ for 24 hours (IFNγ-B cells). Mice received a total of three injections and injections were spaced every two weeks. Cryosections of the right carotid artery containing the largest lesions were used to determine **(A)** VSMCs content (red) using an α-smooth muscle actin staining, **(B)** necrotic core size as by selecting acellular areas in the Masson's trichrome stained sections and calculated as percentage of the total plaque area, and **(C)** CD4^{+} T cell infiltration using a CD4 antibody (green, right), with rat IgG2b isotype (green, left) as control. Data are shown as mean ± SEM. n=5-12/group.

Supplementary table 1. qPCR primers.

Target	Accession #	Forward primer	Reverse primer
CD275 (CD274 antigen, PD-L1)	NM_021893	ggactacaagcgaatcacgctg	tcatgctcagaagtggctggat
Tbx21 (T-box 21, Tbet)	NM_019507	agtgactgcctaccagaacgcagag	ccaggtggcgaggggacact
Bcl6 (B cell leukemia/lymphoma 6)	NM_009744	ctgcaacgaatgtgactgccgtttc	ccgattgaactgcgctccacaaatg
Il6 (interleukin 6)	NM_031168	agacaaagccagagtccttcagaga	ggagagcattggaaattggggtagg
ifng (interferon gamma)	NM_008337	ccttcttcagcaacagcaaggcga	gcgctggacctgtgggttgt
TGF-(b) (transforming growth factor, beta 1, Tgfb1)	NM_011577	agggctaccatgccaacttct	gcaaggaccttgctgtactgtgt
CCR7 (chemokine (C-C motif) receptor 7, EBI-1)	NM_007719	cgtgctggtggtggctctcct	accgtggtattctcgccgatgtagtc
Cxcr5 (chemokine (C-X-C motif) receptor 5)	NM_007551	cgagctgagtggctatctctct	agaggtcactgcggaactttac
Gpr183 (G protein-coupled receptor 183; EBI-2)	NM_183031	ggacgcctgctcgatcactcttaaa	acgttgccagtggggtagtgaaatt
B-actin (actin, beta, Actb)	NM_007393	cttctttgcagctccttcgttgccg	aatacagcccggggagcatcgtc
Rpl27 (Ribosomal protein L27)	NM_011289	cgccaagcgatccaagatcaagtcc	agctgggtccctgaacacatccttg
Rplp0 (Ribosomal protein, large, P0, 36B4)	NM_007475	ctgagtacaccttcccacttactga	cgactcttcctttgcttcagcttt

Supplementary table 2. Antibodies for flow cytometry.

Antigen	Clone	Fluorochrome	Supplier
B220	RA3-6B2	PerCP-Cy5.5	ThermoFisher
B220	RA3-6B2	Alexa fluor 700	ThermoFisher
BCL-6	K112-91	Brilliant Violet 421	BD Biosciences
Blimp-1	5E7	Alexa fluor 647	Biolegend
CD138	281-2	Brilliant Violet 421	Biolegend
CD19	6D5	Brilliant Violet 605	Biolegend
CD21	7G6	Brilliant Violet 421	Biolegend
CD23	B3B4	PE	ThermoFisher
CD279 (PD1)	J43	FITC	ThermoFisher
CD4	RM4-5	FITC	ThermoFisher
CD4	RM4-5	PerCP	ThermoFisher
CD44	IM7	APC	ThermoFisher
CD44	IM7	Brilliant Violet 510	Biolegend
CD45.1	A20	PE	ThermoFisher
CD5	53-7.3	FITC	BD Biosciences
CD62L	MEL-14	FITC	Biolegend
CD8a	53-6.7	FITC	ThermoFisher
CD8a	53-6.7	Alexa fluor 700	Biolegend
CD93	AA4.1	PE-Cy7	ThermoFisher
CD95	Jo2	PE-Cy7	ThermoFisher
CXCR5	L138D7	PE	Biolegend
Fixable Viability Dye		APC-efluor 780	ThermoFisher
FoxP3	FJK-16s	Pacific Blue	ThermoFisher
Gata-3	TWAJ	PE	Biolegend
GL7	GL-7	efluor450	ThermoFisher
ICOS	7E.17G9	PE-Cy7	ThermoFisher
IgD	11-26	APC	ThermoFisher
NK1.1	PK136	FITC	BD Biosciences
PD-L1	10F.9G2	PE-Dazzle 594	Biolegend
PNA		FITC	Vectorlabs
RORγT	Q31-378	Brilliant Violet 650	BD Biosciences
T-bet	eBio4B10	PE-Cy7	ThermoFisher