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## **B cell modulation in atherosclerosis**

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# IFN $\gamma$ -stimulated B cells inhibit T follicular helper cells and protect against atherosclerosis

Submitted

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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 IFN $\gamma$ -stimulated B cells significantly enhanced PD-L1 expression and limited  $T_{FH}$  cell development. To determine whether IFN $\gamma$ -B cells can reduce collar-induced atherosclerosis, *apoE*<sup>-/-</sup> mice fed a Western-type diet were treated with PBS, B cells or IFN $\gamma$ -B cells for a total of 5 weeks following collar placement. IFN $\gamma$ -B cells significantly increased PD-L1<sup>hi</sup> GC B cells and reduced plasmablasts. Interestingly, IFN $\gamma$ -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 IFN $\gamma$ -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 IFN $\gamma$ -stimulated B cells strongly upregulate PD-L1, inhibit  $T_{FH}$  cell responses and protect against atherosclerosis.

## 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<sup>1,2</sup>, 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<sup>3</sup>. 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<sup>4-7</sup>, while in multiple studies B2 cells were seen to contribute to atherosclerosis<sup>8-11</sup>. In addition, the involvement of Th1, Th2 and Treg cells in atherosclerosis has also been examined comprehensively (reviewed in<sup>3</sup>). 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<sup>12</sup>. 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*<sup>-/-</sup>) mice<sup>13</sup> and low-density lipoprotein receptor-deficient mice fed a hypercholesterolemia-inducing diet<sup>14</sup>. In addition, both  $T_{FH}$  cells and FO B cells are proatherogenic and can aggravate atherosclerosis<sup>15 16</sup>.

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.<sup>14</sup> 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<sup>17</sup>. 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.

## 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*<sup>-/-</sup>) 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  $\mu$ l. 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  $\mu$ g/mL), and sedazine (6.25 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*<sup>-/-</sup> mice using CD19<sup>+</sup> 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 (IFN $\gamma$ ; ThermoFisher) for 24 hours. For co-culture experiments, CD4<sup>+</sup> T cells were isolated from splenocytes of C57BL/6 mice using a CD4<sup>+</sup> T cell isolation kit (Miltenyi Biotec). For some experiments, the CD4<sup>+</sup> 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  $\mu$ g/ml) for 72 hours. For adoptive transfer experiments, B cells were isolated from splenocytes of *apoE*<sup>-/-</sup> or CD45.1 mice, cultured in RPMI medium and stimulated for 24 hours with 20 ng/ml of IFN $\gamma$ . 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*<sup>-/-</sup> 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*<sup>-/-</sup> mice were used. *ApoE*<sup>-/-</sup> mice were fed a Western-type diet containing 0.25% cholesterol and 15% cacao butter (SDS, Sussex, UK). For the pilot study, mice were pre-fed a Western-type diet for 2 weeks and were subsequently treated with 2x10<sup>6</sup> freshly isolated or cultured B cells and sacrificed after three days. For atherosclerosis experiments, carotid artery plaque formation was induced after two weeks of Western-type diet feeding by perivascular collar placement in these mice as described previously<sup>35</sup>. Mice continued to be fed a Western-type diet for 5 weeks and during this period received 3 injections with either freshly isolated B cells (2x10<sup>6</sup> cells/injection), B cells stimulated with 20 ng/ml IFN $\gamma$  for 24 hours (2x10<sup>6</sup> 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 IFN $\gamma$ -B cell treated mice were cultured in complete RPMI medium and stimulated for 72 hours with anti-CD3 (1  $\mu$ g/ml) and anti-CD28 (0.5  $\mu$ g/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 (version 10). 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<sup>7</sup>.

### 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). 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). 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 7.0.

## Results

### IFN $\gamma$ -stimulated B cells express high levels of PD-L1

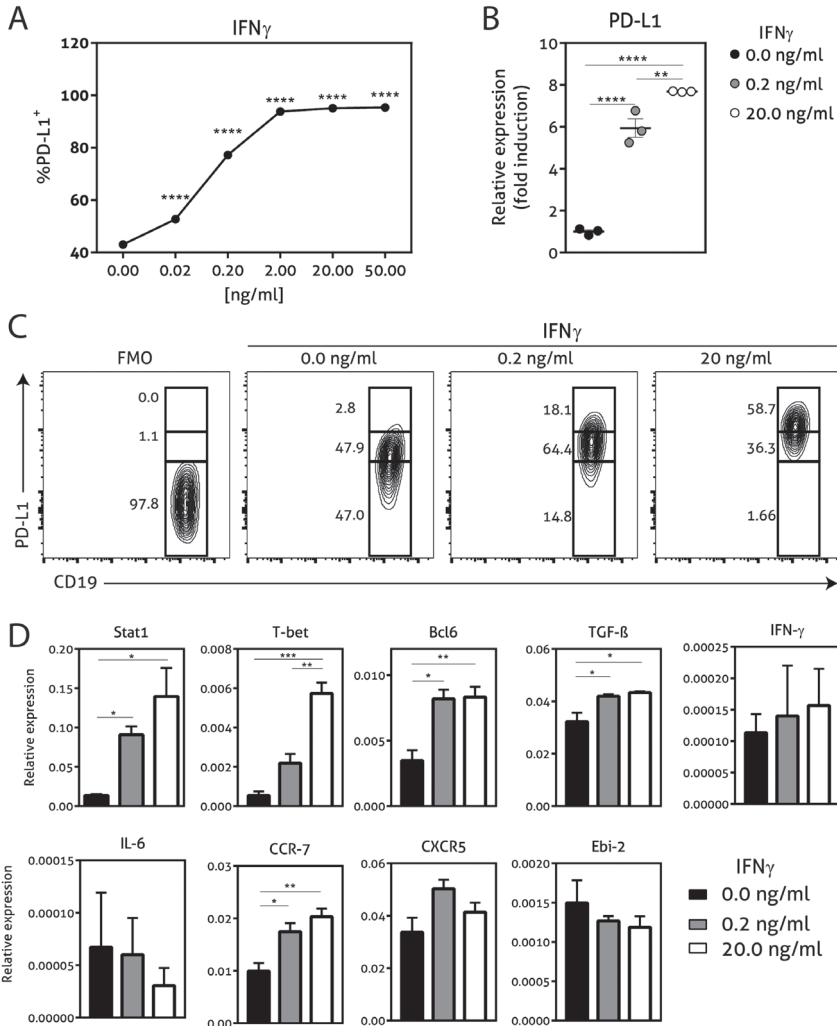
To investigate the role of PD-L1<sup>hi</sup> B cells in atherosclerosis, we first aimed to generate PD-L1<sup>hi</sup> B cells *ex vivo*. We explored several stimuli that previously have been



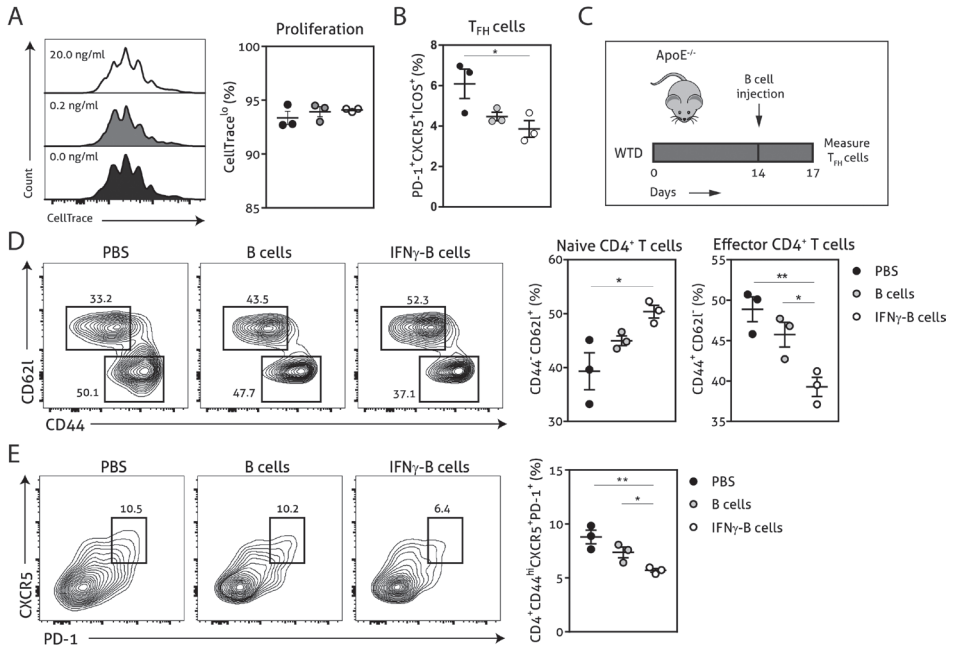
shown to control PD-L1 expression<sup>18,19</sup> (Fig. S1 and Fig 1) and found that IFN $\gamma$  dose-dependently increased the number of PD-L1<sup>+</sup> B cells, with 20.0 ng/ml of IFN $\gamma$  leading to an almost pure population of PD-L1-expressing B cells (Fig. 1A). Using qPCR, we also found an almost 8-fold induction of PD-L1 on mRNA level after IFN $\gamma$  stimulation (Fig. 1B). Furthermore, we observed that the majority of IFN $\gamma$ -stimulated B cells expressed very high levels of PD-L1 (Fig. 1C). Previously, it has been shown that IFN $\gamma$ -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 IFN $\gamma$  and IL-6 production<sup>20,21</sup>. In line with this, our *ex vivo* IFN $\gamma$ -stimulated B cells (IFN $\gamma$ -B cells) showed a similar increase in STAT1, T-bet and BCL-6 (Fig 1D) gene expression. In contrast, we observed a trend towards less IL-6 and no difference in IFN $\gamma$  expression (Fig 1D). Interestingly, IFN $\gamma$  stimulation resulted in a strong significant increase in TGF- $\beta$  expression (Fig. 1D). We also investigated the chemokine receptor profile of these B cells and found a dose-dependent increase in CCR7 expression, while there was no effect on CXCR5 or Ebi-2 (Fig. 1D). 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<sup>22</sup>. Hence, these data indicate that IFN $\gamma$ -stimulated B cells express high levels of PD-L1 and TGF- $\beta$  and a chemokine profile that homes B cells to the T-B cell border.

### IFN $\gamma$ -stimulated B cells inhibit T<sub>FH</sub> cells *in vitro* and *in vivo*

Next, we explored the functional effects of IFN $\gamma$ -stimulated B cells on T<sub>FH</sub> cell development using a CD4<sup>+</sup> T cell and B cell coculture. We stimulated wild-type CD4<sup>+</sup> T cells for 72 hours with anti-CD3 in the presence of unstimulated or IFN $\gamma$ -stimulated *apoE*<sup>-/-</sup> B cells. Although we did not observe any difference in proliferative capacity of CD4<sup>+</sup> T cells (Fig. 2A), we found a remarkable decrease of T<sub>FH</sub> cells when CD4<sup>+</sup> T cells were cocultured with IFN $\gamma$ -stimulated B cells compared to unstimulated B cells (Fig 2B). These findings illustrate that IFN $\gamma$ -B cells are able to curb T<sub>FH</sub> cell development *in vitro*. We subsequently tested IFN $\gamma$ -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 IFN $\gamma$  for 24 hours into *apoE*<sup>-/-</sup> mice. To induce initial T<sub>FH</sub> cell accumulation, mice were fed a Western-type diet for two weeks before they received the adoptive transfers (Fig. 2C)<sup>14</sup>. We observed a remarkable reduction in effector CD4<sup>+</sup> T cells in mice treated with IFN $\gamma$ -B cells compared to mice that received PBS or B cells (Fig. 2D). Contrary, the number of naïve CD4<sup>+</sup> T cells was increased in IFN $\gamma$ -B cells treated mice compared to mice receiving PBS (Fig. 2D). Most importantly, treatment of mice with IFN $\gamma$ -B cells resulted in a strong reduction in T<sub>FH</sub> cells compared to mice that were administered with PBS or B cells (Fig. 2E). These data demonstrate that IFN $\gamma$ -B cells are also able to inhibit T<sub>FH</sub> cells *in vivo*.



**Figure 1. Characterization of IFN $\gamma$ -stimulated B cells.** CD19<sup>+</sup> B cells were isolated from C57BL/6 mice and (A) stimulated for 24 hours with different doses interferon-gamma (IFN $\gamma$ ) after which PD-L1 protein expression was measured with flow cytometry. (B) CD19<sup>+</sup> B cells were unstimulated or stimulated with 0.2 ng/ml or 20.0 ng/ml IFN $\gamma$  for 24 hours, after which mRNA expression of PD-L1 was assessed using qPCR. (C) B cells as stimulated in (B) were analyzed for PD-L1<sup>lo</sup>, PD-L1<sup>int</sup> and PD-L1<sup>hi</sup> expression with flow cytometry. (D) mRNA expression was analyzed for depicted genes in B cells as stimulated in (B). Data are analyzed with a One-Way ANOVA and shown as mean  $\pm$  SEM (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.00001$ ).  $n = 3/\text{group}$ .

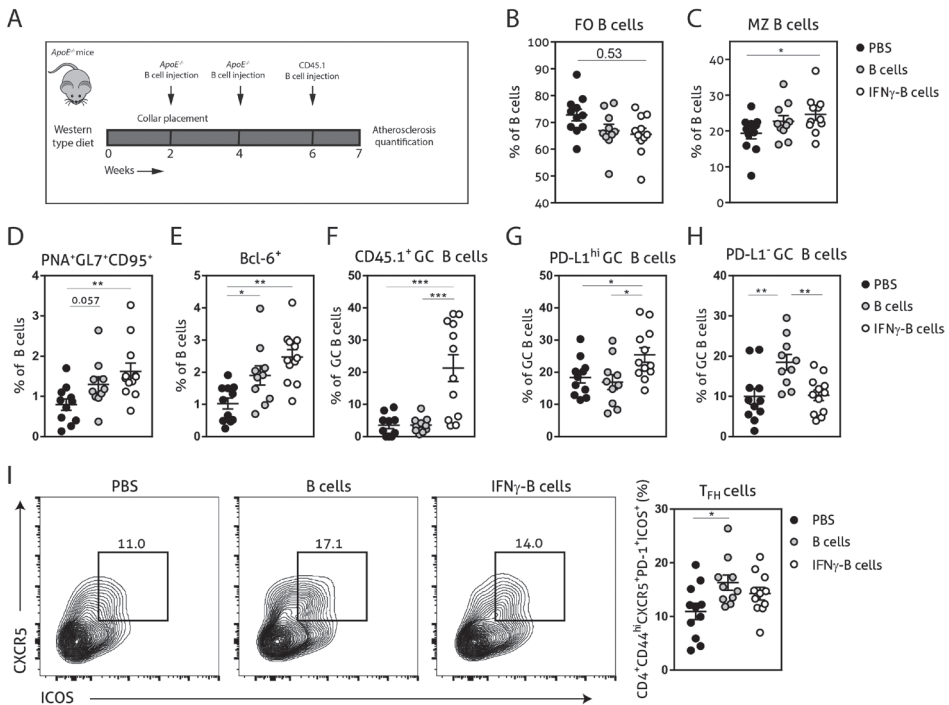


**Figure 2. IFN $\gamma$ -B cells inhibit T follicular helper cells *in vitro* and *in vivo*.** Isolated CD19<sup>+</sup> B cells were unstimulated or stimulated with 0.2 ng/ml or 20.0 ng/ml IFN $\gamma$  for 24 hours and co-cultured with isolated CD4<sup>+</sup> T cells. The coculture was stimulated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

### Adoptive transfer of IFN $\gamma$ -B cells during atherosclerosis development affects the T<sub>FH</sub> – GC B cell axis

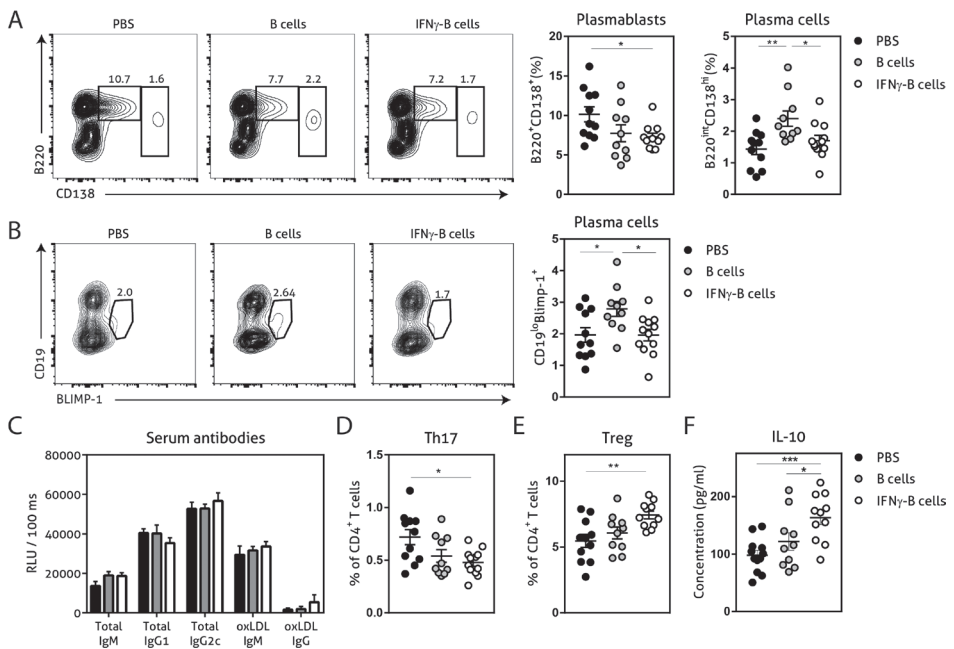
Given the *in vitro* and *in vivo* regulatory effects of IFN $\gamma$ -B cells on T<sub>FH</sub> cells, we further investigated whether these B cells would be able to restrict T<sub>FH</sub> cell numbers during atherosclerosis development. We fed *apoE*<sup>-/-</sup> mice a Western-type diet for 2 weeks after which we placed a perivascular collar and started treatment with either PBS, B cells or IFN $\gamma$ -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<sub>FH</sub> – GC B cell axis. IFN $\gamma$ -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 IFN $\gamma$ -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 IFN $\gamma$ -B cells indeed reached the germinal center (Fig. 3F) and locally increased the number of PD-L1<sup>hi</sup> 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<sup>-</sup> (Fig. 3H). At sacrifice, we did not observe a difference in T<sub>FH</sub> cells in IFN $\gamma$ -B cell-treated mice, while adoptive transfer of B cells resulted in an increase in T<sub>FH</sub> cells compared to PBS-treated mice (Fig. 3I).



**Figure 3. Adoptive transfer of IFN $\gamma$ -B cells during atherosclerosis development affects the T<sub>FH</sub> - GC B cell axis.** (A) *ApoE*<sup>-/-</sup> 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 IFN $\gamma$  for 24 hours (IFN $\gamma$ -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<sup>+</sup> germinal center B cells, (G) PD-L1<sup>hi</sup> germinal center B cells, (H) PD-L1<sup>-</sup> germinal center B cells and (I) flow charts and quantification of follicular helper T cells (CD44<sup>hi</sup>PD-1<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup>). Data are analyzed with a One-Way ANOVA and shown as mean  $\pm$  SEM (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).  $n = 10-12$ /group.

Next, we assessed the number of plasmablasts and plasma cells and found a significant reduction in plasmablasts when mice received IFN $\gamma$ -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 IFN $\gamma$ -B cells-treated mice. Since BLIMP-1 is the driving transcription factor for plasma cell generation<sup>23</sup>, we also measured BLIMP-1 expression which revealed a significant increase in BLIMP-1<sup>+</sup> cells in mice that received B cells compared to mice receiving PBS or IFN $\gamma$ -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).



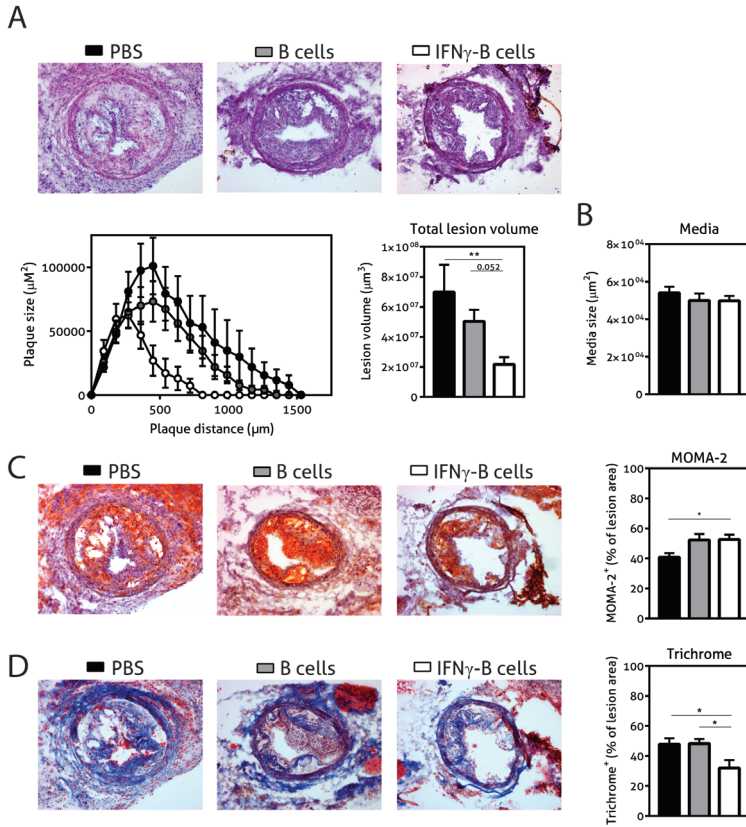
**Figure 4. Effects of adoptive transfer of IFN $\gamma$ -B cells on the humoral immunity.** *ApoE*<sup>-/-</sup> mice were fed a Western type diet for 7 weeks. After 2 weeks they received a perivascular collar and were treated with PBS, freshly isolated B cells (B cells) or B cells stimulated with 20.0 ng/ml IFN $\gamma$  for 24 hours (IFN $\gamma$ -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<sup>+</sup>CD138<sup>+</sup>) and plasma cells (B220<sup>int</sup>CD138<sup>hi</sup>). (B) Flow charts and quantification of CD19<sup>lo</sup>BLIMP-1<sup>+</sup> plasma cells. (C) Serum was analyzed for circulating antibodies by ELISA. Spleens were analyzed with flow cytometry for (D) Th17 cells (ROR $\gamma$ t<sup>+</sup>) or (E) Treg cells (FoxP3<sup>+</sup>). (F) Splenocytes were isolated and stimulated with anti-CD3 (1  $\mu$ g/ml) and anti-CD28 (0.5  $\mu$ g/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  $\pm$  SEM (\*  $p$ <0.05, \*\*  $p$ <0.01).  $n$ =10-12/group.

### Adoptive transfer of IFN $\gamma$ -B cells promotes an anti-inflammatory CD4<sup>+</sup> T cell response

The CD4<sup>+</sup> T cell response is also highly involved in the pathogenesis of atherosclerosis and PD-L1<sup>hi</sup> B cells have previously shown to restrict CD4<sup>+</sup> T cell differentiation<sup>17</sup>. While we did not observe any differences in Th1 or Th2 CD4<sup>+</sup> T cells between mice treated with IFN $\gamma$ -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 IFN $\gamma$ -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 IFN $\gamma$ -B cells treated mice compared to splenocytes from mice treated with PBS or B cells (Fig. 4F).

### Adoptive transfer of IFN $\gamma$ -B protects against lesion formation

Given the immune-regulating effects of IFN $\gamma$ -B cells, we subsequently assessed whether adoptive transfer of these IFN $\gamma$ -B cells was able to reduce collar-induced atherosclerosis in *apoE*<sup>-/-</sup> mice. We quantified lesion development in the carotid arteries and found a significant reduction in total lesion volume when mice were treated with IFN $\gamma$ -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 IFN $\gamma$ -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 IFN $\gamma$ -B cells inhibited atherosclerotic lesion progression towards more advanced lesions.



**Figure 5. Adoptive transfer of IFN $\gamma$ -B protects against atherosclerosis.** *ApoE*<sup>-/-</sup> 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 IFN $\gamma$  for 24 hours (IFN $\gamma$ -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 analyzed using a Trichrome staining. Data are analyzed with a One-Way ANOVA and shown as mean  $\pm$  SEM (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).  $n = 8-12$ /group.

## Discussion

Up to date, three groups have reported the proatherogenic role of the T<sub>FH</sub> – GC B cell axis<sup>13-15</sup>. Direct depletion of T<sub>FH</sub> cells in *Ldlr*<sup>-/-</sup> mice resulted in a reduction of atherosclerosis<sup>15</sup>, and loss of control on the T<sub>FH</sub> – GC B axis by depletion of MZ B cells<sup>14</sup> or CD8<sup>+</sup> regulatory T cells<sup>13</sup> aggravated atherosclerosis. We now show that adoptive transfer of PD-L1 expressing B cells inhibit T<sub>FH</sub> cell responses both *in vitro* and *in vivo* and protect against atherosclerosis.

Using *ex vivo* stimulation of B cells with IFN $\gamma$  we rapidly provided a large and almost pure population of PD-L1-expressing B cells. IFN $\gamma$  is an extensively investigated cytokine with a broad range of immune actions. Interestingly, while we show here that IFN $\gamma$  stimulation of B cells results in a regulatory phenotype that reduces the number of T<sub>FH</sub> cells, earlier work identified a role for IFN $\gamma$  in the generation of spontaneous germinal centers and B cell autoreactivity<sup>20,24</sup>. These previous data were, however, mainly acquired in the context of autoimmunity, where B cells receive a multitude of different signals and IFN $\gamma$  signaling seems primarily to synergize with BCR-, CD40- and TLR-mediated stimuli to induce spontaneous germinal centers<sup>24</sup>. Under these circumstances, IFN $\gamma$  signaling in B cells results in increased IL-6 and IFN $\gamma$  production which drives auto-immunity<sup>20,21</sup>. In contrast, our *ex vivo* stimulated B cells lacked additional stimuli and upregulated expression of PD-L1 and TGF- $\beta$ , while we did not see any effects on IL-6 and IFN $\gamma$ . We further showed that IFN $\gamma$ -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*<sup>-/-</sup> mice interact with pre-T<sub>FH</sub> cells at the T-B cell border in response to a high-cholesterol diet<sup>14</sup>. During our atherosclerosis study, we indeed found that adoptively transferred IFN $\gamma$ -B cells showed the characteristics of B cells that reside in or near the germinal center.

Similar to flow-sorted PD-L1<sup>hi</sup> B cells<sup>17</sup>, we next showed that IFN $\gamma$ -B cells were able to inhibit T<sub>FH</sub> cell numbers *in vitro* and *in vivo*. This study further demonstrated that IFN $\gamma$ -B cells were able to promote anti-inflammatory CD4<sup>+</sup> T cells. This corresponds with the effects found in experimental autoimmune encephalomyelitis using PD-L1<sup>hi</sup> B cells, which restricted Th1 and Th17 differentiation<sup>17</sup>. Along this line, we found that IFN $\gamma$ -B cells resulted in decreased Th17 cells and a significant increase in atheroprotective Tregs and IL-10 production of CD4<sup>+</sup> T cells.

Furthermore, we demonstrated atheroprotective effects of IFN $\gamma$ -B cells that express high levels of PD-L1, which 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<sup>32</sup>. Similarly, PD-1 knockout mice or mice treated with a PD-1 blocking antibody developed exacerbated atherosclerosis<sup>33,34</sup>. Our data is further supported by previous studies that demonstrated the proatherogenic role of T<sub>FH</sub> cells<sup>13-15</sup>. However, due to the pleiotropic nature of IFN $\gamma$ , other factors besides PD-L1 could also have contributed to our findings. Indeed, the observed increase in TGF- $\beta$  expression could have contributed to both the T<sub>FH</sub> inhibition and Treg induction, since TGF- $\beta$  signaling is known to prevent T<sub>FH</sub> cell accumulation and B cell autoreactivity<sup>25</sup>. In addition, there have been a large number of studies with adoptive



transfer of B cells expressing TGF- $\beta$  that reported immune tolerance in autoimmune mouse models<sup>26-29</sup>. 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 IFN $\gamma$ -B cells. PD-L1 is also known to be essential for the induction of Treg cells<sup>30</sup>, the Treg accumulation could thus be a combined effect of increased TGF- $\beta$  and PD-L1 expression by IFN $\gamma$ -B cells. Since the atheroprotective effects of Treg cells is well characterized<sup>31</sup>, the observed Treg induction undoubtedly contributed to the reduced atherosclerosis found with adoptive transfer of IFN $\gamma$ -B cells.

In conclusion, this study uncovers a new role for *ex vivo* stimulation of B cells with IFN $\gamma$  for the induction of atheroprotective B cells. IFN $\gamma$ -B cells show the genetic makeup of GC B cells with increased expression of PD-L1 and TGF- $\beta$  and effectively inhibit T<sub>FH</sub> cells *in vitro* and *in vivo* and ameliorate atherosclerosis development in *apoE*<sup>-/-</sup> mice. These results further emphasize the proatherogenic role of the T<sub>FH</sub> - GC B axis and provide a novel way to regulate this axis.

## Acknowledgements

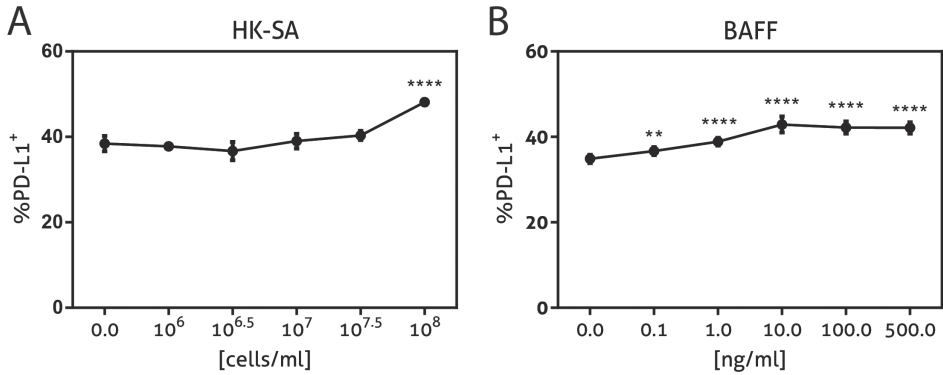
We thank Maria Ozsvar Kozma for her technical assistance with the antibody measurements.

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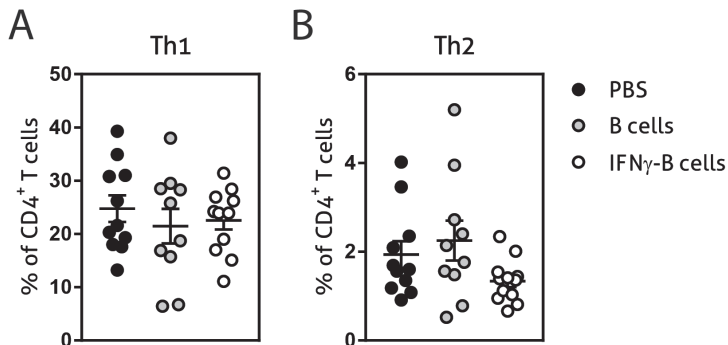
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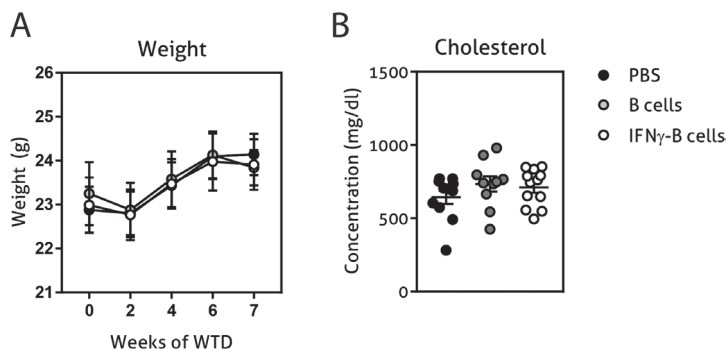
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**Supplementary Figure 1.** PD-L1 expression of stimulated B cells. CD19<sup>+</sup> B cells were isolated from C57BL/6 mice and stimulated for 24 hours with different doses of (A) heat killed *S. Aureus* (HK-SA) or (B) B cell activating factor (BAFF) after which PD-L1 expression was measured with flow cytometry. Data are analyzed with a One-Way ANOVA and shown as mean  $\pm$  SEM (\*\*  $p < 0.01$ , \*\*\*\*  $p < 0.0001$ ).  $n = 3$ /group.



**Supplementary Figure 2.** Effects of adoptive transfer of IFN $\gamma$ -B cells on Th1 and Th2 cells. *ApoE*<sup>-/-</sup> 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 IFN $\gamma$  for 24 hours (IFN $\gamma$ -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<sup>+</sup>) and (B) Th2 cells (Gata-3<sup>+</sup>). Data are shown as mean  $\pm$  SEM.  $n = 10-12$ /group.



**Supplementary Figure 3.** *ApoE*<sup>-/-</sup> 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 IFN $\gamma$  for 24 hours (IFN $\gamma$ -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.

**Table 1**

Target	Accession #	Forward primer	Reverse primer
CD274 (CD274 antigen, PD-L1)	NM_021893	ggactacaagcgaatcacgctg	tcagtctcagaagtggctggat
Tbx21 (T-box 21, Tbet)	NM_019507	agtgactgcctaccagaacgcagag	ccaggtggcaggggagact
Bcl6 (B cell leukemia/lymphoma 6)	NM_009744	ctgcaacgaatgtgactgccgtttc	ccgattgaactgcgctccacaatg
Il6 (interleukin 6)	NM_031168	agacaaagccagagtccttcagaga	ggagagcaltggaattggggtagg
infg (interferon gamma)	NM_008337	cctcttcagcaacagcaaggcga	gcgctggacctgtgggtgt
TGF-(b) (transforming growth factor, beta 1, Tgfb1)	NM_011577	agggctaccatccaacttct	gcaaggaccttctgtactgtgt
CCR7 (chemokine (C-C motif) receptor 7, EBI-1)	NM_007719	ctgtctggtggtgctctctct	accgtgtattctgccgatgtagtc
Cxcr5 (chemokine (C-X-C motif) receptor 5)	NM_007551	cgagctgagtggtctatctctct	agaggtcactgcggaactttac
Gpr183 (G protein-coupled receptor 183; EBI-2)	NM_183031	ggacgcctgctgatcactcttaaa	acgttgcagtggggtagtgaatt
B-actin (actin, beta, Actb)	NM_007393	cttcttgagctccttcgttgccg	aatacagccgggagcatcgtc
Rpl27 (Ribosomal protein L27)	NM_011289	cgccaagcatccaagatcaagtcc	agctgggtccctgaacacatcctg
Rplp0 (ribosomal protein, large, P0, 36B4)	NM_007475	ctgagtacacctcccactactga	cgactctctttgcttcagcttt

Table 2

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	BV510	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	ThermoFisher
GL7	GL-7	eFluor 450	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
RORyt	Q31-378	Brilliant Violet 650	BD Biosciences
T-bet	eBio4B10	PE-Cy7	ThermoFisher

