

Follicular regulatory helper T cells control the response of regulatory B cells to a high-cholesterol diet

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Received 25 January 2019; revised 14 October 2019; editorial decision 7 March 2020; accepted 23 March 2020; online publish-ahead-of-print 19 April 2020

Time for primary review: 37 days

Aims

B cell functions in the process of atherogenesis have been investigated but several aspects remain to be clarified.

Methods and results

In this study, we show that follicular regulatory helper T cells (T_{FR}) control regulatory B cell (B_{REG}) populations in $Apoe^{-/-}$ mice models on a high-cholesterol diet (HCD). Feeding mice with HCD resulted in up-regulation of T_{FR} and B_{REG} cell populations, causing the suppression of proatherogenic follicular helper T cell (T_{FH}) response. T_{FH} cell modulation is correlated with the growth of atherosclerotic plaque size in thoracoabdominal aortas and aortic root plaques, suggesting that T_{FR} cells are atheroprotective. During adoptive transfer experiments, T_{FR} cells transferred into HCD mice decreased T_{FH} cell populations, atherosclerotic plaque size, while B_{REG} cell population and lymphangiogenesis are significantly increased.

Conclusion

Our results demonstrate that, through different strategies, both T_{FR} and T_{FH} cells modulate anti- and pro-atherosclerotic immune processes in an $Apoe^{-/-}$ mice model since T_{FR} cells are able to regulate both T_{FH} and B_{REG} cell populations as well as lymphangiogenesis and lipoprotein metabolism.

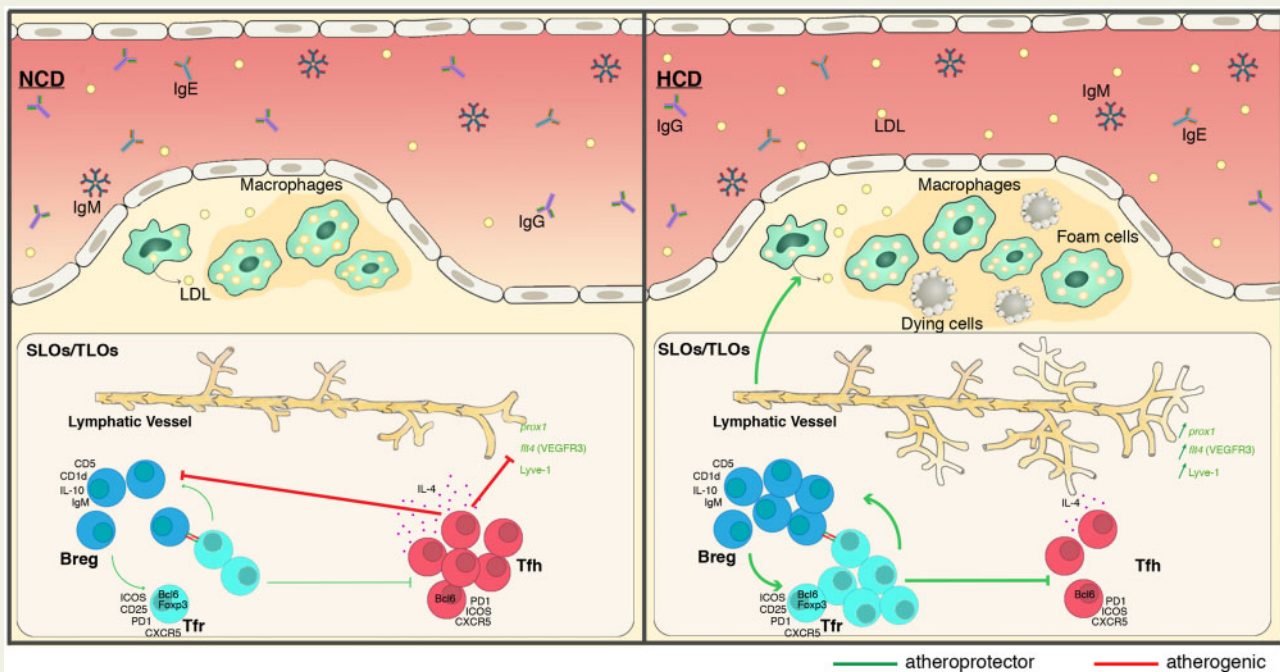
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Graphical Abstract



Keywords

Follicular helper T cell (T_{FH}) • Follicular regulatory helper T cells (T_{FR}) • Regulatory B cell • Lymphangiogenesis • Atherosclerosis

1. Introduction

Cardiovascular diseases (CVD) are the main cause of deaths worldwide. One of the most studied CVD is atherosclerosis. Its development is characterized by the activation of innate and adaptive immune responses to high concentrations of circulating lipids. During this process, T and B cells are significantly involved, although the exact functions of T and B cell subtypes are not fully understood. $Foxp3^+$ regulatory T cells (T_{REG}) assume an immunosuppressive function by reducing experimental atherosclerosis.¹ It has been paradoxically demonstrated that atherogenesis is associated with increasing T_{REG} cell populations while their depletion accelerates atherosclerosis and promotes the atherogenic lipoprotein profile.^{1–3} The recently identified follicular regulatory helper T cell (T_{FR}) populations are a subset of T_{REG} cells localized in germinal centre (GC) where they control the magnitude of the response by suppressing T_{FH} and B cell activation.⁴ Since the size and specificity of the GC is modulated by T_{FR} cells, GC responses represent an important axis for atherogenesis regulation.⁴ It has been demonstrated very recently that follicular helper T cells (T_{FH}) are proatherogenic, and innate-like marginal zone B cells (MZB) participate in their regulation.^{5,6} The study of B cell subsets indicates that B_{REG} cell populations are up-regulated in $Apoe^{-/-}$ mice and carry atheroprotective functions, confirming that other subsets of B cells could also participate in atherogenesis.⁷

T_{REG} cells have been shown to modulate cholesterol homeostasis, and $CD4^+$ T cells have been identified as regulators of lymphatic vessel formation.^{8,9} Whereas the lymphatic system has long been described to be involved in immune cell trafficking,¹⁰ recent studies suggest that it is a

prerequisite player in macrophage reverse cholesterol transport (RCT).¹¹ Early atherosclerotic plaque formation is associated to a defect in the capacity of the collecting lymphatic vessel to propel lymph,¹² whereas advanced lesion is also associated to a defect in the absorption of lymph from peripheral tissues.^{11,13} Furthermore, defective lymphatic function appears to have a systemic effect on B cell homing and to impair lipoprotein metabolism that promotes atherosclerosis.¹⁴ The present study clearly identifies T_{FR} cells as key regulators of atherosclerosis through decreasing T_{FH} cell populations, thereby inducing expansion of anti-atherogenic B_{REG} cell populations and stimulating lymphangiogenesis.

2. Methods

2.1 Ethical statement

All breeding and experimental protocols and procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Geneva University School of Medicine (protocol number: GE/16/56 and GE/16/131). Animal care and experimental procedures were carried out in accordance with the guidelines of the Institutional Animal Care, Use Committee of the Geneva University School of Medicine and complied with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

2.2 Mice

An 11-week-old $Apoe^{-/-}$ C57Bl/6 mice was submitted to two atherogenesis protocols to test the Bcl-6 inhibitor 79-6 (Merck, Germany). In the

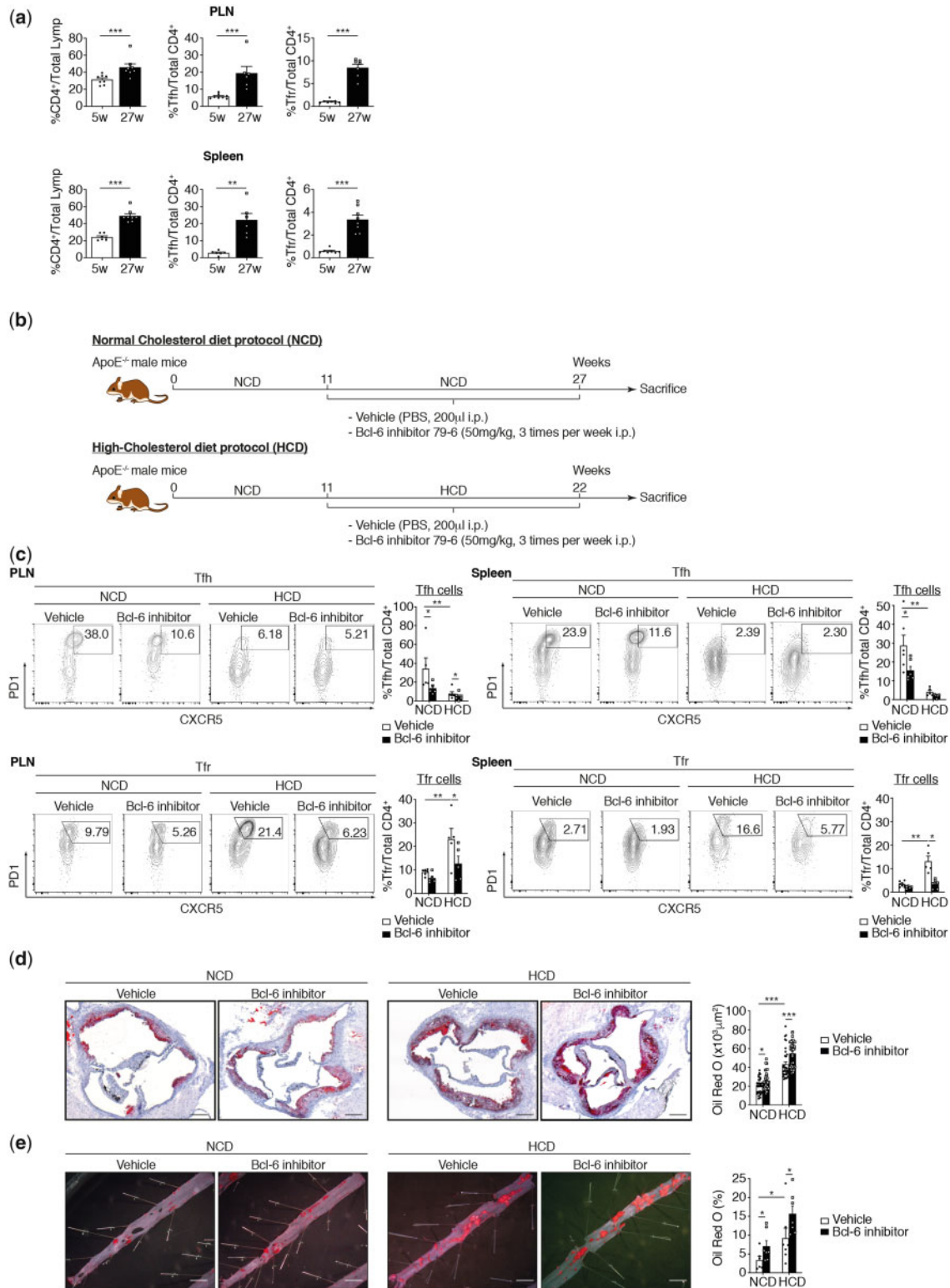


Figure 1 T_{FR} population controls pro-atherogenic T_{FH} population on a high-cholesterol diet. (A) Lymphocytes from spleen and peripheral lymph nodes (PLN) of ApoE^{-/-} mice collected at Weeks 5 and 27 were stained for CD4, CD25, CXCR5, PD1, and Bcl-6. Tabulated results from all mice are presented as percentage of CD4⁺. (B) Mouse atherosclerosis protocols and treatment schedule ($n = 5-8$ mice/group). (C) Effect of Bcl-6 inhibitor on T_{FH} and T_{FR} populations on NCD and HCD fed mice (PLN T_{FH}: $n = 5-8$ mice/group; PLN T_{FR}: $n = 5-6$ mice/group; spleen T_{FH}: $n = 5-6$ mice/group; spleen T_{FR}: $n = 5-8$ mice/group). (D) Quantification of atherosclerotic lipid content and representative microphotographs of aortic root plaques (Oil Red O staining) in ApoE^{-/-} mice treated with vehicle (PBS) or Bcl-6 inhibitor under NCD and HCD ($n = 6-7$ mice per group/5 slides per mice). Original magnification, $\times 10$. Scale bars, 400 μm . (E) Quantification of atherosclerotic lipid content and representative microphotographs from Oil Red O-stained thoracoabdominal aortas ($n = 5-7$ mice/group). Scale bars, 500 μm . The non-parametric Mann-Whitney U test was used for statistical analysis: * $P \leq 0.05$; ** $P \leq 0.005$; *** $P \leq 0.0005$. All data were represented as mean \pm SEM.

earlier atherosclerosis protocol, mice were fed a normal chow diet (NCD) for 16 weeks (Figure 1B). In the more advanced atherosclerosis protocol, to accelerate atherogenesis, mice were fed a high-cholesterol diet (HCD) for 11 weeks (20.1% fat, 1.25% cholesterol, Research Diets, Inc., New Brunswick, NJ, USA) (Figure 1B).¹⁰ Mice were randomly assigned to receive treatments either with vehicle (10% DMSO) or Bcl-6 inhibitor 79-6. This drug (10 mg/mL, diluted in 200 μ L 10% DMSO per injection corresponding to 50 mg/kg) and vehicle (equal volume of 200 μ L 10% DMSO) were intraperitoneally injected three times per week before animal euthanasia by exsanguination following the guidelines of the Institutional Animal Care. No analgesia or anaesthesia was required before these intraperitoneally injections. Mice well-tolerated the treatments and atherosclerosis protocols and no adverse events (such as weight loss and signs of systemic toxicity) were reported. At sacrifice, haematological parameters, serum triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C), free fatty acids, and glucose were routinely measured and expressed in mmol/L. Animals were sacrificed by exsanguination after anaesthesia with 4% isoflurane.

2.3 Flow cytometry

For nine-colour immunofluorescence analysis, single-cell suspensions (1×10^6 cells) of spleen and lymph nodes (inguinal, mesenteric, brachial, axillary, and superficial cervical) were incubated with anti-mouse FcR1B/FcR1IA mAb (BD Biosciences, MD, USA) to avoid non-specific staining and were subsequently stained with fluorophore-conjugated antibodies (see Supplementary material online, Table S1) at 4°C using predetermined optimal concentrations of mAb for 30 min. Briefly, lymphocytes were stimulated *in vitro* with PMA (50 ng/mL; Sigma-Aldrich, Germany) and ionomycin (1 μ g/mL; Sigma-Aldrich, Germany), in the presence of brefeldin A (1 μ L/mL; Sigma-Aldrich, Germany) for 4 h before staining. After staining, cells were washed, fixed, and then permeabilized using the Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences, MD, USA) according to the manufacturer's instructions. Permeabilized cells were then stained with antibodies against intracellular targets of interest. FACS data will be acquired in a Gallios™ flow cytometer (BD Biosciences, MD, USA) and analysed using FlowJo software (TreeStar, Version 10.0.8r1). For analysis, dead cells and doublets were excluded based on exclusion dye or forward scatter profiles, respectively. T_{FR} cells were gated as CD4⁺Foxp3⁺CD25⁺PD1⁺CXCR5⁺, while T_{FH} cells were gated as CD4⁺Foxp3⁻CD25⁻PD1⁺CXCR5⁺. Then, cell populations have analysed for their Bcl-6 expression (T_{FR} and T_{FH} cell population) and IL-21 expression (T_{FH} cell population) (see Supplementary material online, Figure S1A). B_{REG} cells were gated as B220⁺CD43⁻CD1d^{high}CD5⁺, follicular B cells as B220⁺CD43⁻CD21⁺CD23⁺ and MZB as B220⁺CD43⁻CD21⁺CD23⁻CD5⁻. Then, MZ, FO, and B_{REG} cell population have analysed for their IL-10 and IgM expression, respectively (see Supplementary material online, Figure S2A). All positive cells were defined using a 'fluorescence minus one' (FMO) sample.

2.4 Aorta single-cell preparation

The aorta was perfused and, then, cut into pieces before being digested using a cocktail of enzymes for 60 min at 37°C (1.2 mg/mL collagenase P and Dispase I, and 180 U/mL DNase I). Then, aorta single-cell suspension has been subjected to flow cytometry protocol.

2.5 Immunohistochemistry

Mouse aortic sinus was serially cut in 5 μ m transversal sections, as previously described.^{15,16} Sections from mouse specimens were fixed in acetone and immunostained with specific antibodies anti-mouse CD68 (macrophages, ABD Serotec, Düsseldorf, Germany), anti-mouse Ly-6G (neutrophils, BD Pharmingen™, CA, USA), anti-mouse MMP-9 (R&D Systems, MN, USA), or LYVE-1 (Abcam, Cambridge, UK). Vector Red alkaline phosphatase substrate: SK-5100; in association with Levamisole solution: SP-5000; which produce a magenta colouration is used for revelations (Vector Laboratories, Inc., CA, USA). Quantifications were performed using the MetaMorph or Definiens software. Results for other parameters were calculated as percentages of stained area on total lesion area, number of infiltrating cells per mm² of lesion area or number of lymphatic vessels in adventitia.

2.6 Oil Red O staining for lipid content

Five sections per mouse aortic and aortas sinus were stained with Oil Red O, as previously described.^{15,16} Sections and aortas were counter-stained with Mayer's hemalun and rinsed in distilled water. Quantifications were performed using the MetaMorph software. Data were calculated as ratios of stained area on total lesion area.

2.7 Quantitative real-time PCR

Total mRNA was prepared by Trizol® (ThermoFischer), according to the provider protocol. Reverse transcription was performed using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Real-time PCR (StepOne Plus, Applied Biosystems, Waltham, MA, USA) was performed with the SensiFast (LabGene). Real-time duplex qPCR analysis was conducted as described elsewhere.^{17,18} The levels of mRNA expression were normalized against the expression of a housekeeping gene (*hprt*) and analysed using the comparative Δ CT method. Probes were purchased from Applied Biosystems. All measurements were conducted in triplicate.

2.8 Adoptive transfer

Adoptive transfers were performed as previously described.¹⁹ Briefly, recipient mice received two intraperitoneal injections of single-cell follicular regulatory helper T cell suspensions collected from three donor mice. Briefly, T cells from spleens and lymph nodes (inguinal, mesenteric, brachial, axillary, and superficial cervical) were isolated by passing the tissue through 70 μ m cell strainer (BD Biosciences, MD, USA). Erythrocytes were lysed and nucleated cells washed twice, counted, and suspended in a culture medium. T cell suspensions were enriched with CD4⁺CD25⁺ regulatory T cell isolation kit (Miltenyi Biotec, Germany) and sorted with Beckman Coulter MoFlo Astrios (CD4⁺CD25⁺PD1⁺CXCR5⁺) under sterile conditions (1.5×10^5 /injection/mouse) (see Supplementary material online, Figure S1B). Dead cells and doublets were excluded based on exclusion dye or forward scatter profiles, respectively. T cell purity (>99%) and phenotype were confirmed by flow cytometry. For T_{FR} homing experiments, T_{FR} was stained with Yellow trace™ (ThermoFisher, MA, USA) before their intraperitoneal injections.

2.9 In vitro B cell differentiation assay

Follicular regulatory helper T cells from spleens and LN collected from three Apoe^{-/-} donor mice were enriched with CD4⁺CD25⁺ regulatory T cell isolation kit (Miltenyi Biotec) and sorted with Beckman Coulter MoFlo Astrios (CD4⁺CD25⁺PD1⁺CXCR5⁺) under sterile conditions

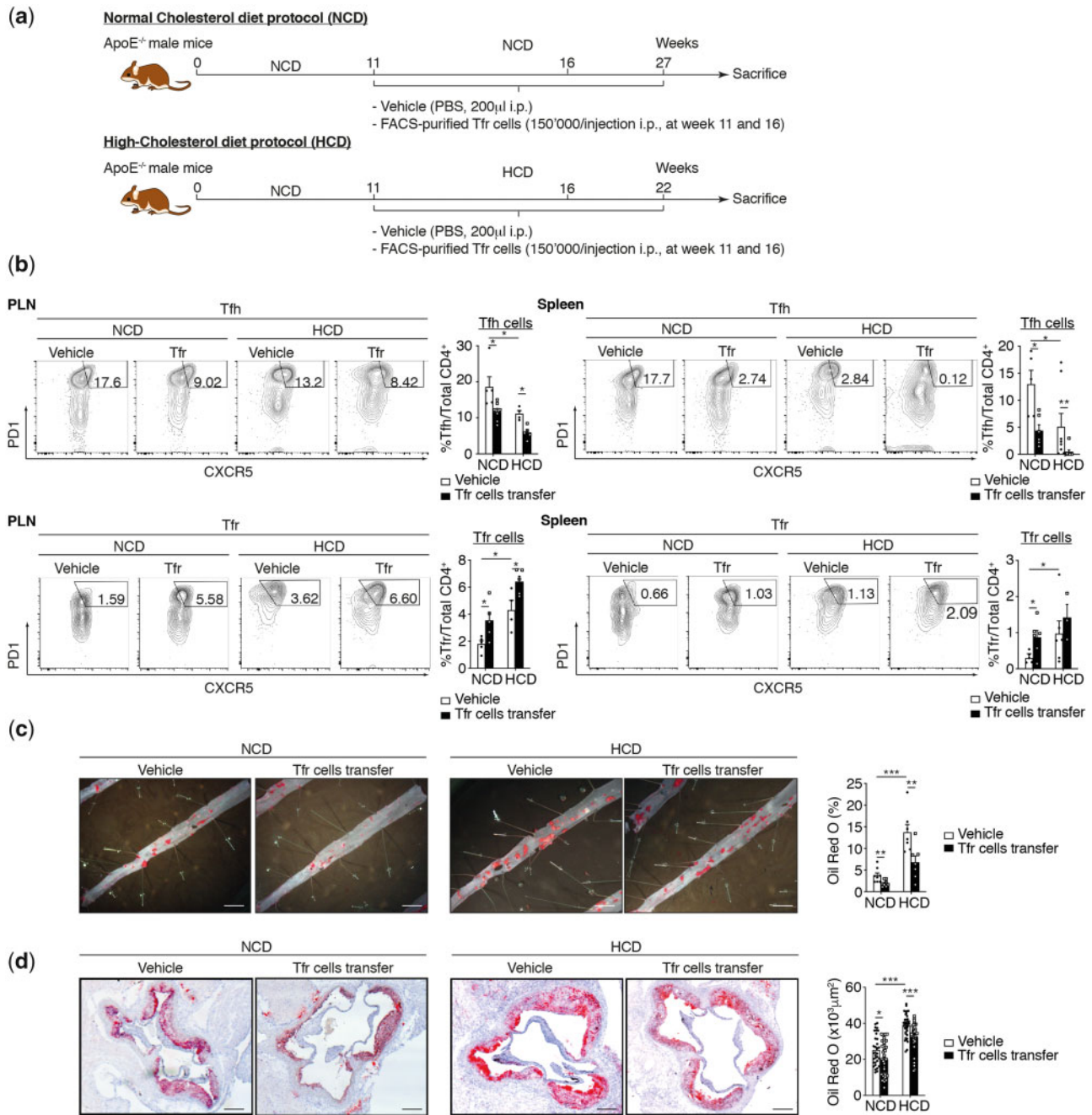


Figure 2 T_{FR} cell population regulates atherogenesis in HCD. (A) Mouse atherosclerosis protocols and adoptive transfer strategy. (B) Effect of T_{FR} cell transfer on secondary lymphoid organs T_{FH} and T_{FR} populations on NCD and HCD fed mice (PLN T_{FH}: $n = 4-7$ mice/group; PLN T_{FR}: $n = 4-5$ mice/group; spleen T_{FH}: $n = 4-8$ mice/group; spleen T_{FR}: $n = 3-6$ mice/group). (C) Quantification of atherosclerotic lipid content and representative microphotographs from Oil Red O-stained thoracoabdominal aortas in ApoE^{-/-} mice treated with vehicle (PBS) or T_{FR} cells (T_{FR} cell transfer) under NCD and HCD ($n = 5-8$ mice/group). (D) Quantification of atherosclerotic lipid content and representative microphotographs of aortic root plaques (Oil Red O staining) in ApoE^{-/-} mice treated with vehicle (PBS) or T_{FR} cells (T_{FR} cell transfer) under NCD and HCD ($n = 7$ or 8 mice per group/5 slides per mice). Scale bars, 500 μm. Original magnification, $\times 10$. Scale bars, 400 μm. The non-parametric Mann-Whitney U test was used for statistical analysis: * $P \leq 0.05$; ** $P \leq 0.005$; *** $P \leq 0.0005$. All data were represented as mean \pm SEM.

(see [Supplementary material online, Figure S1A](#)). Dead cells and doublets were excluded based on exclusion dye or forward scatter profiles, respectively. Follicular helper T cells were isolated from flow-through from CD4⁺CD25⁺ regulatory T cell isolation kit, which was then

positively selected and sorted (CD4⁺CD25⁺PD1⁺CXCR5⁺). Single-cell suspension followed by purification with the Pan B Cell Isolation Kit (Miltenyi Biotec) was performed in order to obtain purified B cells from ApoE^{-/-} mice. *In vitro* suppression assays were performed as described.²⁰

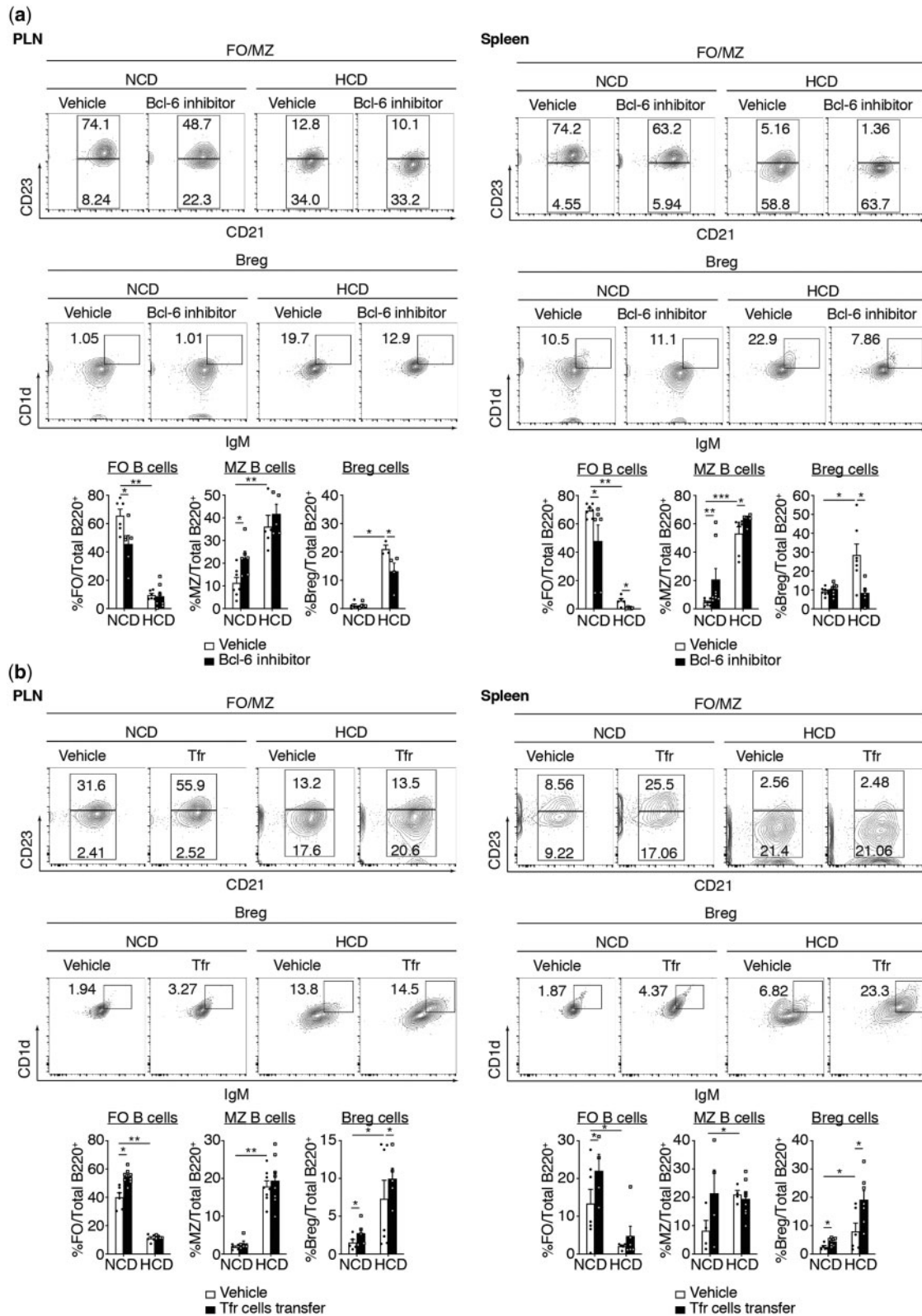


Figure 3 T_{FR} cells increase B_{REG} and FO B cell population. (A) Effect of Bcl-6 inhibitor on secondary lymphoid organs B cell populations on an NCD and HCD fed mice (PLN FO B: *n* = 6 mice/group; PLN MZB: *n* = 5–7 mice/group; PLN B_{REG}: *n* = 3–5 mice/group; spleen FO B: *n* = 5–7 mice/group; spleen MZB: *n* = 5–8 mice/group; spleen B_{REG}: *n* = 5–8 mice/group). (B) Effect of T_{FR} cell transfer on secondary lymphoid organs B cell populations on NCD and HCD fed mice (PLN FO B: *n* = 5–8 mice/group; PLN MZB: *n* = 5–8 mice/group; PLN B_{REG}: *n* = 5–7 mice/group; spleen FO B: *n* = 4–7 mice/group; spleen MZB: *n* = 4–8 mice/group; spleen B_{REG}: *n* = 5–7 mice/group). The non-parametric Mann–Whitney *U* test was used for statistical analysis: **P* ≤ 0.05; ***P* ≤ 0.005; ****P* ≤ 0.0005. All data were represented as mean ± SEM.

Briefly, 5×10^4 B cells, 3×10^4 T_{FH} cells, and/or $750\text{--}5 \times 10^4$ T_{FR} cells were plated in 96-well plates along with 2 µg/mL anti-CD3 (145-2c11, eBioscience) and 5 µg/mL anti-IgM (FFA21, Invitrogen). For analysis, B_{REG} cells were gated as B220⁺CD43⁻IgM^{high}CD1d^{high}CD5⁺, follicular B cells as B220⁺CD43⁻CD21⁺CD23⁺ and MZB as B220⁺CD43⁻CD21⁺CD23⁻CD5⁻. Cell supernatants were harvested, diluted twice, and used to treat purified B cells from Apoe^{-/-} mice. Briefly, 5×10^4 B cells were plated in 96-well plates along with 5 µg/mL anti-IgM (FFA21, Invitrogen) and treated with cells supernatant. For analysis, B_{REG} cells were analysed as described above.

2.10 Statistical analysis

Data are presented as mean ± SEM. For clinical scores, significance between groups was analysed using the non-parametric Mann–Whitney *U* test because values were not normally distributed and/or the population size was too small ($n \leq 8$). * $P \leq 0.05$; ** $P \leq 0.005$; *** $P \leq 0.0005$.

3. Results

3.1 T_{FR} cell population controls the pro-atherogenic T_{FH} cell population in Apoe^{-/-} mice on an HCD

Recent publications have shown that T_{FH} cell populations are important for the T_{FH}-cell germinal-centre response to an HCD.^{5,6} It has thus been demonstrated that T_{FH} cell populations expand during the development of atherosclerosis while MZB negatively regulate T_{FH} cells. Both studies suggest that T_{FH} cells are a pro-atherogenic cell population. As T_{FR} cells naturally regulate T_{FH} cells, we investigated the potential anti-atherogenic function of T_{FR} cell populations. The gating strategy described in [Supplementary material online, Figure S1A](#), was used to characterize T_{FH} and T_{FR} cell populations. Positive cells were defined using a FMO sample. We initially considered the spontaneous evolution of T_{FH} and T_{FR} cells in Apoe^{-/-} mice from 5 to 27 weeks. While we observed that CD4⁺ overall and T_{FH} cell populations in particular increase in secondary lymphoid organs in adult Apoe^{-/-} mice on a normal cholesterol diet as previously described,⁶ we also observed an expansion of T_{FR} cell populations ([Figure 1A](#)). The frequency of T_{FH} cell populations is, however, higher than that of T_{FR} cell populations ([Figure 1A](#)). To address the role of T_{FR} cells in atherosclerosis, we used two atherogenesis protocols based on earlier and more advanced phases of the disease.^{21,22} In the earlier atherosclerosis protocol, mice were fed an NCD for 16 weeks to avoid the onset of severe hypercholesterolaemia. To accelerate atherogenesis in the more advanced atherosclerosis protocol, mice were fed a HCD for 11 weeks ([Figure 1B](#)). In parallel, we used Bcl-6 transcriptional repressor (Bcl-6 inhibitor 79-6) to inhibit T_{FR} and T_{FH} cell differentiation. In Apoe^{-/-} mice on HCD, T_{FH} cell populations significantly decreased while T_{FR} cell populations increased leading to sustained changes in the ratio between T_{FR} and T_{FH} cells in secondary lymphoid organs ([Figure 1C](#)). In this context, Bcl6 inhibitor injected three times per week for the last 16 weeks (NCD) or 11 weeks (HCD) strongly abrogated T_{FR} and T_{FH} cell differentiation ([Figure 1C](#)). Bcl6 inhibitor seems to have also some effects on Th2 and Th17 but not on Th1 in secondary lymphoid organs. Indeed, Th2 cell population is decreased in Bcl6 inhibitor-treated mice in both diet while Th17 seems to be decreased only in spleen of HCD-fed mice (see [Supplementary material online, Figure S2A](#)). The disruption of T_{FH} and T_{FR} cell populations in peripheral lymph nodes (PLN) and the spleen induced by Bcl6 inhibitor markedly accelerated both early

and advanced atherosclerotic lesion sizes in thoracoabdominal aortas and aortic root as compared to untreated mice ([Figure 1D and E](#)). These results suggest that the pro-atherogenic role of T_{FH} cells is less essential than the anti-atherogenic function of T_{FR} cells in the process of atherogenesis. It further implies that regulation of T_{FH} cell populations is not the only function of T_{FR} cells.

As additional functions of T_{FR} cells have not previously been described, we therefore performed an adoptive transfer approach in order to gain a mechanistic insight. After sorting T_{FR} cells from 27-week-old Apoe^{-/-} mice, we adoptively transferred these cells twice into Apoe^{-/-} recipient mice, at 11 and 16 weeks ([Figure 2A](#) and see [Supplementary material online, Figure S1B](#)). We observed that T_{FR} cells reach secondary lymphoid organs and aorta at 3 days. They are still present 3-week post-injection (see [Supplementary material online, Figure S1C and D](#)). In Apoe^{-/-} mice under NCD and HCD, the increase of T_{FR} cell frequency and the significant reduction of T_{FH} cell populations brought about by adoptive transfer ([Figure 2B](#)) abrogates the acceleration both of early and of advanced atherosclerotic lesion size in thoracoabdominal aortas and aortic root as compared to vehicle-treated mice ([Figure 2C and D](#)). In both early and advanced atherosclerotic, the adoptive transfer of T_{FR} cells leads also to decrease Th1 and Th2 cell population in spleen while Th2 and Th17 cells are modulated only in lymph nodes (see [Supplementary material online, Figure S2B](#)). These data demonstrate that abrogation of both T_{FH} and T_{FR} cell populations leads to the exacerbation of atherosclerotic lesions, whereas T_{FR} cells display atheroprotective properties. This, in turn, means that discarding T_{FH} cell populations diminishes atherogenesis only if T_{FR} cell populations remain present.

3.2 T_{FR} cells control B_{REG} cell differentiation in presence of T_{FH} cells

T_{FR} cells regulate T_{FH} cell populations while also playing some pleiotropic functions. In this context, T_{FH} cells are known to provide both co-stimulation and stimulatory cytokines for B cell differentiation, leading to the development of GCs.²³ In order to determine the additional roles of T_{FR} cells in atherogenesis, we investigated the role of T_{FR} cells in B cell differentiation in an early and advanced atherosclerosis mouse model. Using the gating strategy described in [Supplementary material online, Figure S3A](#), we characterized three potentially important B cell subtypes: follicular B cell (FOB), MZB, and B_{REG}. We observed a marked augmentation of MZB and B_{REG} cell populations in secondary lymphoid organs of mice in advanced atherosclerotic model, while FOB populations and serum IgG1 level decrease ([Figure 3A and B](#) and see [Supplementary material online, Figure 3B](#)). Bcl6 inhibitors have only a weak effect on FOB and MZB populations in advanced atherosclerosis. In the early atherosclerosis model, Bcl6 inhibitors both diminished and increased the frequencies of FOB and MZB population, respectively ([Figure 3A](#)). However, Bcl6 inhibitor induces a strong increase of IgG1, IgM, and IgE in both atherosclerotic models (see [Supplementary material online, Figure S3B](#)). Interestingly, Bcl6 inhibitor abrogated B_{REG} cell-augmented frequency brought about by an HCD ([Figure 3A](#)) while their ability to produce IL-10 is not modulated neither by an HCD nor by an Bcl6 inhibitor (data not shown). As recent data have indicated that B_{REG} cells may have anti-atherogenic properties,⁷ we used adoptive transfer of T_{FR} cells ([Figure 2A](#)) to evaluate whether B_{REG} cell populations are regulated by T_{FR} cells. We observed that T_{FR} cell transfer induces an expansion of B_{REG} cell populations in advanced atherosclerosis while other B cell subsets are not affected ([Figure 3B](#)). T_{FR} cell transfer triggers a significant

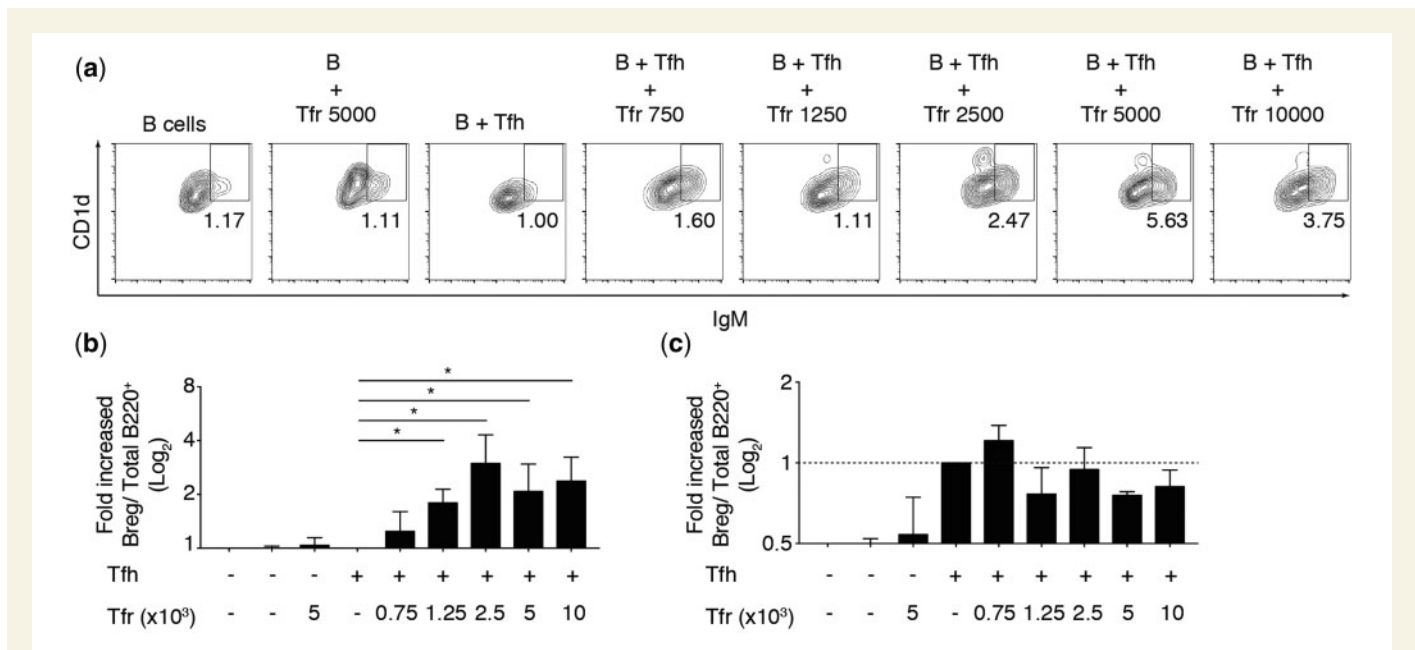


Figure 4 T_{FR} cells control B_{REG} cell differentiation *in vitro*. (A) Representative flow cytometry plots of the effects of dose response of T_{FR} cell number on *in vitro* B_{REG} cell differentiation in the presence of T_{FH} cells. (B) Quantification of B_{REG} cell differentiation relative to initial B_{REG} cell population and expressed in fold increased ($n = 5$). (C) Quantification of B_{REG} cell differentiation induced by cell supernatant from *in vitro* differentiation relative to initial B_{REG} cell population and expressed in fold increase ($n = 5$). The non-parametric Mann–Whitney *U* test was used for statistical analysis: * $P \leq 0.05$. Data were represented as mean \pm SEM.

augmentation of IgM and a diminution of IgE in serum of NCD- and HCD-fed mice (see [Supplementary material online, Figure S3C](#)). However, T_{FR} cell transfer increases B_{REG} cells but also FOB populations in early atherosclerosis (Figure 3B). Our data strongly suggest that T_{FR} cells regulate B_{REG} cell populations. We next examined whether T_{FR} cells are also able to regulate the expansion of B_{REG} cell populations *in vitro*. Following the protocol of Sage *et al.*,²⁰ we demonstrated that T_{FR} cells do control B_{REG} cell expansion *in vitro* in presence of T_{FH} cells. B_{REG} cell populations indeed increase proportionally to T_{FR} cell numbers when T_{FH} cells are present (Figure 4A and B), providing direct evidence that T_{FR} cells regulate B_{REG} cell populations. T_{FR} cells also increased FOB populations, although to a lesser extent than B_{REG} cells, but not MZB populations (see [Supplementary material online, Figure S4A](#)). The regulation of B_{REG} cell expansion by T_{FR} cells requires direct contact between both of them. *In vitro* stimulation of B cells with the supernatant from a differentiation assay, in fact, had no effect on B_{REG} cell proliferation or differentiation (Figure 4C). Our results established that T_{FR} cells regulate B_{REG} cell populations both *in vivo* and *in vitro*. Our findings further suggest that the ability of T_{FR} cells to trigger B_{REG} cell expansion in advance atherosclerosis contributes to their anti-atherogenic properties.

3.3 T_{FR} cells regulate lymphangiogenesis in advanced atherosclerosis

Intriguingly, while the disruption of T_{FH} and T_{FR} cell populations in PLN and the spleen, induced by Bcl6 inhibitors, markedly accelerated both early and advanced atherosclerotic lesion sizes in the aortic root as compared to untreated mice (Figure 1E), the amount of infiltrated macrophages in the aortic root was not affected (Figure 5A). However, the increase of T_{FR} cell frequency and the considerable reduction of T_{FH} cell

populations brought about by adoptive transfer (Figure 2B) triggered a significant reduction of infiltrated macrophages (Figure 5B). The relative proportions of macrophage subsets within a plaque could be also an indicator of plaque stability. We thus investigated the M1 and M2 polarization of aortic roots macrophages upon T_{FR} cells adoptive transfer. We have quantified the M1 and M2 subsets population simultaneously based on inducible nitric oxide synthase for M1 macrophages and Arginase-1 for M2 macrophages (see [Supplementary material online, Figure S5A](#)).^{24,25} While M1 macrophages polarization seems to be not affected by HCD, M2 macrophages prominently decreased in HCD-fed mice. T_{FR} adoptive cell transfer do not have significant effects on M1 or M2 macrophages neither in NCD nor in HCD (see [Supplementary material online, Figure S5B and C](#)). In this context, the emigration of mononuclear phagocytes or efferocytosis through lymphatic vessels to secondary lymphoid organs, that normally characterizes the resolution of inflammation, is impaired during atherogenesis.²⁶ Furthermore, we observed that lymphatic vessel dysfunction and degeneration present in hypercholesterolaemic mice alters the lipid balance and plasma cholesterol levels.^{26,27} Finally, it became clear that CD4 T cells and IL-4 regulate lymphatic vessel formation, and that the main producer of IL-4 in GC is the T_{FH} cell population.^{8,9,28–30} We next considered whether T_{FR} cells control lymphangiogenesis and, in turn, the plasmatic lipid balance by inhibiting T_{FH} cell-dependent IL-4 production. To examine this, we investigated the expression of prospero homeobox 1 (*prox1*), lymphatic vessel endothelial hyaluronan receptor 1 (*lyve1*), vascular endothelial growth factor receptor 3 (*flt4*), and IL-4 (*Il4*) in the spleens of both Bcl-6-inhibitor and T_{FR}-treated Apoe^{-/-} mice fed with an NCD or an HCD. We observed in advance, atherosclerosis (not in early), a repression of *prox1*-, *lyve1*-, and *flt4*-mRNA expression, is associated to an advanced atherosclerotic lesion, thereby demonstrating lymphangiogenesis inhibition in the late

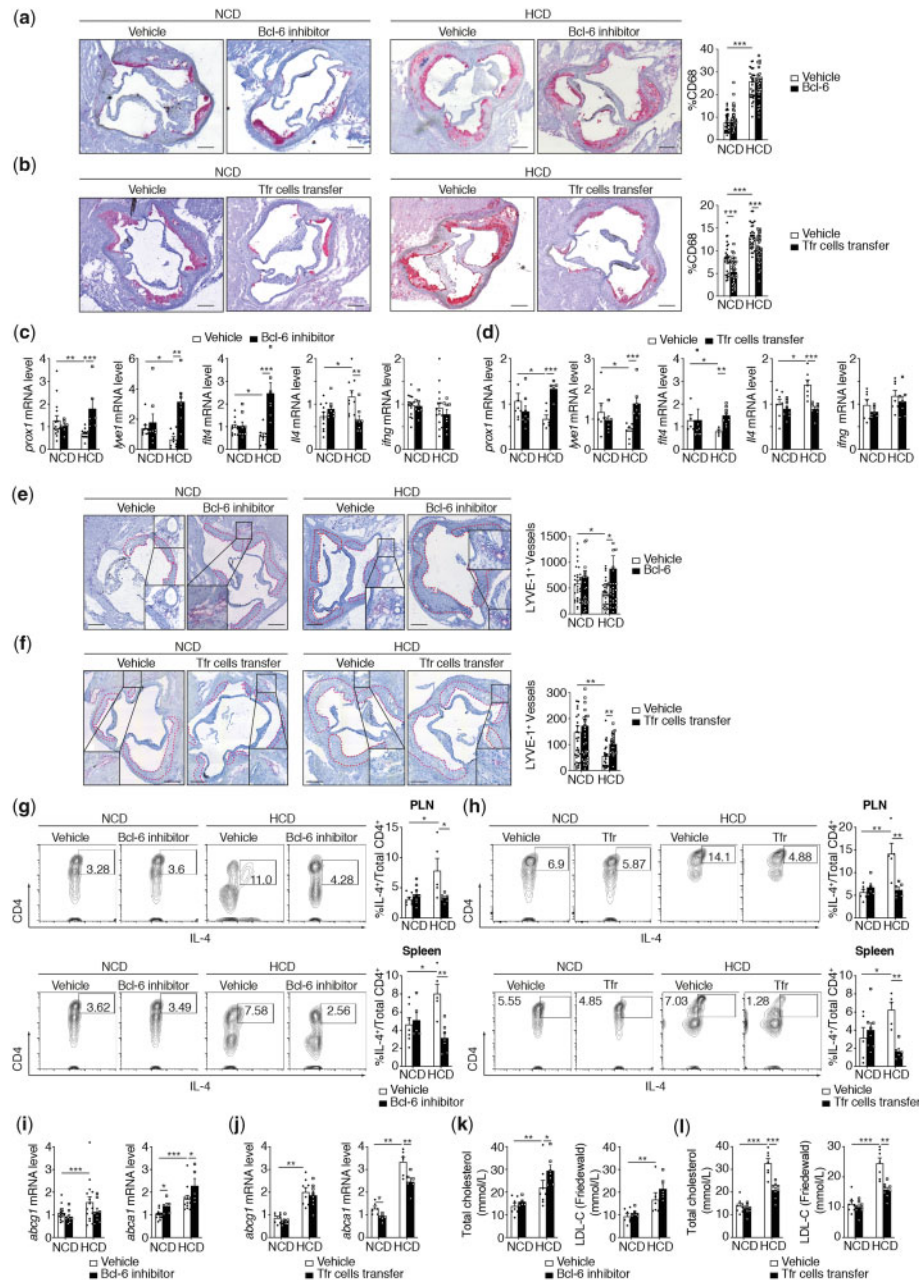


Figure 5 T_{FR} cells control lymphangiogenesis through IL-4 secretion. (A) Quantification of CD68 expression and representative microphotographs of aortic root plaques in *Apoe*^{-/-} mice treated with vehicle (PBS) or Bcl-6 inhibitor under NCD and HCD ($n = 6-7$ mice per group/5 slides per mice). (B) Quantification of CD68 expression and representative microphotographs of aortic root plaques in *Apoe*^{-/-} mice treated with vehicle (PBS) or T_{FR} cells (T_{FR} cell transfer) under NCD and HCD ($n = 6-7$ mice per group/5 slides per mice) Original magnification, $\times 10$. Scale bars, 400 μm . (C) Effect of Bcl-6 inhibitor on mRNA expression of lymphangiogenic regulators in the spleen under NCD and HCD fed mice (*prox1*: $n = 6-15$ mice/group; *lyve1*: $n = 7-10$ mice/group; *flt4*: $n = 7-11$ mice/group; *il4*: $n = 8-11$ mice/group; *ifng*: $n = 8-15$ mice/group). (D) Effect of T_{FR} cells transfer on mRNA expression of lymphangiogenic regulators in the spleen of NCD and HCD fed mice (*prox1*: $n = 5-8$ mice/group; *lyve1*: $n = 6-7$ mice/group; *flt4*: $n = 4-8$ mice/group; *il4*: $n = 7-8$ mice/group; *ifng*: $n = 6-7$ mice/group). (E) Quantification and representative microphotographs of the number of LYVE-1⁺ lymphatic vessels in *Apoe*^{-/-} mice treated with vehicle (PBS) or Bcl-6 inhibitor under NCD and HCD. Red dotted line surround the plaques ($n = 8$ mice per group/5 slides per group). (F) Quantification and representative microphotographs of the number of LYVE-1⁺ lymphatic vessels in *Apoe*^{-/-} mice treated with vehicle (PBS) or T_{FR} cells (T_{FR} cell transfer) under NCD and HCD. Red dotted line surround the plaques ($n = 8$ mice per group/5 slides per mice). (G) Effect of Bcl-6 inhibitor on secondary lymphoid organs CD4⁺IL-4⁺-producing cells in NCD and HCD fed mice (PLN IL-4: $n = 5-8$ mice/group; spleen IL-4: $n = 5-7$ mice/group). (H) Effect of T_{FR} cell transfer to secondary lymphoid organs CD4⁺IL-4⁺-producing cells in NCD and HCD feed mice (PLN IL-4: $n = 5-6$ mice/group; spleen IL-4: $n = 4-8$ mice/group). (I) Effect of Bcl-6 inhibitor on mRNA expression of reverse cholesterol efflux regulators (*abcg1* and *abca1*) on the spleen of NCD and HCD fed mice ($n = 5-15$ mice/group). (J) Effect of T_{FR} cell transfer on mRNA expression of reverse cholesterol efflux regulators (*abcg1* and *abca1*) in the spleen of NCD and HCD fed mice ($n = 6-8$ mice/group). (K) Effect of Bcl-6 inhibitor on serum level of total cholesterol and LDL-c (Friedewald) in NCD and HCD fed mice ($n = 5-8$ mice/group). (L) Effect of T_{FR} cell transfer on serum level of total cholesterol and LDL-c (Friedewald) in NCD and HCD fed mice ($n = 7-8$ mice/group). The non-parametric Mann-Whitney *U* test was used for statistical analysis: * $P \leq 0.05$; ** $P \leq 0.005$; *** $P \leq 0.0005$. All data were represented as mean \pm SEM.

stage of the disease (Figure 5C and D). *Ii4* mRNA expression was inversely increased in advance atherosclerosis. Treatment with Bcl-6 inhibitors or T_{FR} cells led to a strong increase in *prox1*-, *lyve1*-, and *flt4*-mRNA expression and concomitantly to an important reduction in *Ii4*-mRNA expression. Although IFN γ could also modulate lymphangiogenesis, its mRNA levels were not affected in advance atherosclerosis (Figure 5C and D). Consistently, the number of LYVE-1⁺ vessels in adventitia is decreased in advanced lesions while Bcl-6 inhibitors or T_{FR} cell transfer restore the lymphangiogenesis as demonstrated by the increase in the number of LYVE-1⁺ vessels in adventitia (Figure 5E and F). CD4⁺IL-4⁺ cells are clearly and consistently increased by an HCD in secondary lymphoid organs. Bcl-6 inhibitors and T_{FR} cell adoptive transfer inhibit non-Th2-cell CD4⁺IL-4⁺ populations (Figure 5G and H and see Supplementary material online, Figure S6A and B).

The lymphatic system is involved in cholesterol homeostasis. Martel et al.¹¹ have specifically shown that the lymphatic vessel route is crucial for RCT, independently of the efflux capacity of cholesterol from macrophages. We, however, examined whether T_{FH} and T_{FR} cell depletion, as well as T_{FR} cell adoptive transfer, could modulate other steps of the RCT process, an observed their effect on ATP-binding cassette transporters ABCA1 and ABCG1, which in turn are involved in cholesterol efflux. Our data indicate that *abca1*- and *abcg1*-mRNA expression are increased in the spleen in advance atherosclerosis, although neither Bcl-6 inhibitors nor transferred T_{FR} cells modulate the ABCA1 gene (Figure 5I and J). On the other hand, in both NCD- and HCD-fed mice, *abca1* mRNA expression is up-regulated when Bcl-6 inhibitors deplete T_{FH} and T_{FR} cells, whereas it is restrained when T_{FR} cells are transferred (Figure 5I and J). These effects could be also observed with the liver X receptors- α (LXR α) gene (see Supplementary material online, Figure S6C and D). In Apoe^{-/-} mice fed an NCD or HCD, levels of plasmatic LDL-C, calculated using the Friedewald equation, and total cholesterol follow the same profiles as *abca1* gene expression (Figure 5K and L). These data suggest that T_{FH} cells regulate lymphangiogenesis through IL-4 secretion. The results further demonstrate that reduction of both T_{FH} and T_{FR} cell populations leads to up-regulation of RCT-involved genes, whereas T_{FR} cells decrease RCT genes. Results also indicated that T_{FR} cell populations regulate plasma cholesterol levels.

3.4 T_{FR} cell population is not able to repair established atherosclerosis lesions

T_{FR} cell populations carry atheroprotective functions as demonstrated by adoptive transfer (Figure 2C and D). We, therefore, examined the potential atheroregression properties of T_{FR} cells. We used the same protocol as that described previously but this time Apoe^{-/-} mice were fed an HCD for 11 weeks and then switched to an NCD before twice-adoptive transferring T_{FR} cells (Figure 6A). T_{FR} cells transferred to Apoe^{-/-} mice with established atherosclerotic lesions had no significant impact on the size of lesions present in root and abdominal aorta (Figure 6B and C). However, the frequency of T_{FR} and T_{FH} cells in secondary lymphoid organs was increased and decreased by the adoptive transfer of T_{FR} cells, respectively (Figure 6D). As regards the B cell population, we observed that T_{FR} cell transfer increased both B_{REG} and FOB cell populations while MZ B cells were not affected. T_{FR} cell transfer triggers a significant augmentation of IgM and a diminution of IgE in the serum of mice (see Supplementary material online, Figure S6E). These data indicate that T_{FR} cell populations are involved in the regulation of atherogenesis through a dampening of the progression of atherosclerotic lesions. Similarly, reducing T_{FH} cell populations following the establishment of

atherosclerotic lesions has no effect on lipid deposition. Altogether, these results show that T_{FR} cell populations cannot be considered as a means of treating established atherosclerosis. Instead, it may be worth evaluating the use of T_{FR} cells in the primary setting for patients with familial hypercholesterolaemia.

4 Discussion

The important role played by specific B and T cell populations with regard to the adaptive immune response in atherosclerosis appears to be markedly greater than previously established. In fact, it was previously shown that MZB cells, T_{FH} cells, as well as CD8 regulatory T cell populations, can modulate atherosclerosis.^{5,6} To our knowledge, the function of T_{FR} cells within this context has not been previously investigated. Our results suggest that T_{FR} cell populations play a crucial role in the establishment of atherosclerotic lesions in response to an HCD.

T_{FH} cells clearly constitute a crucial cell population involved in atherogenesis. It has in fact recently been shown that T_{FH} cells carry atherogenic properties and that their defective regulation characterizes atherogenesis.^{5,6} Consistently, our results have shown a high T_{FH}/T_{FR} cell ratio in young and adult Apoe^{-/-} mice fed on an NCD, while an increase in T_{FH} cell regulation by adoptive transferred T_{FR} cells strongly limits plaque development in an HCD. Nus et al.⁵ have also demonstrated that T_{FH} cells have proatherogenic functions and that their frequency is regulated by MZB cells when subjected to an HCD. These findings raise the question of mutual regulation of T and B cells in the T_{FH}-GC B cell axis. Our data suggest that B_{REG} cell populations are strongly increased in advanced atherosclerosis. Adoptive transfer of T_{FR} cells reinforces this growth and, *in vitro*, a differentiation assay of B cells confirmed that T_{FR} cells control B_{REG} differentiation. However, T_{FR}-dependent differentiation of B_{REG} cells required direct contact between both of them, as demonstrated by the disability/inability of a supernatant from *in vitro* differentiation assay to trigger B_{REG} differentiation. Both B cell populations (MZB and B_{REG} cell populations) possess CD1d molecules on their cell surface.^{31,32} CD1 proteins belong to a family of major histocompatibility complexes that present lipid molecules or hydrophobic peptide antigens to T cells.³³⁻³⁵ It appears that the uptake of antigenic lipids by CD1-positive DCs might facilitate cell activation, while macrophages take up lipid antigen and degrade it.³⁶ This is consistent with the antigen-presenting cell properties of B cells especially since these cells have regulatory functions in atherogenesis.

While the regulatory mechanisms operating within the follicles prevent autoreactivities they do enable the selection of high-affinity antibody-producing cells. In this context, regulatory B cells correspond to B lymphocytes that control the proportion and function of T and B effector cells. It has been revealed that B_{REG} cells are increased in hypercholesterolaemic mice and protect from lesion development.⁷ Achour et al.³⁷ have further demonstrated that human B_{REG} cells control T_{FH} cell maturation, expand T_{FR} cells, and inhibit T_{FH} cell-mediated antibody secretion. Our findings indicate that T_{FR} cells control B_{REG} cell populations. It thereby seems that B_{REG} and T_{FR} cell populations are mutually self-regulated, leading to an amplification loop of regulation dampening T_{FH} cell frequency and, in turn, atherogenesis. Consistently, while T_{FH} cell populations are the main producers of IL-4 in GC, IL-4 is able to indirectly inhibit B_{REG} cell expansion.³⁸ IL-4 thus seems to play a crucial function in atherogenesis. It has been clearly demonstrated that double knockout mice, Apoe^{-/-}/IL-4^{-/-}, show a 58% decrease in plaque size in the aortic arch compared to Apoe^{-/-} mice.³⁹ In this context, the anti-

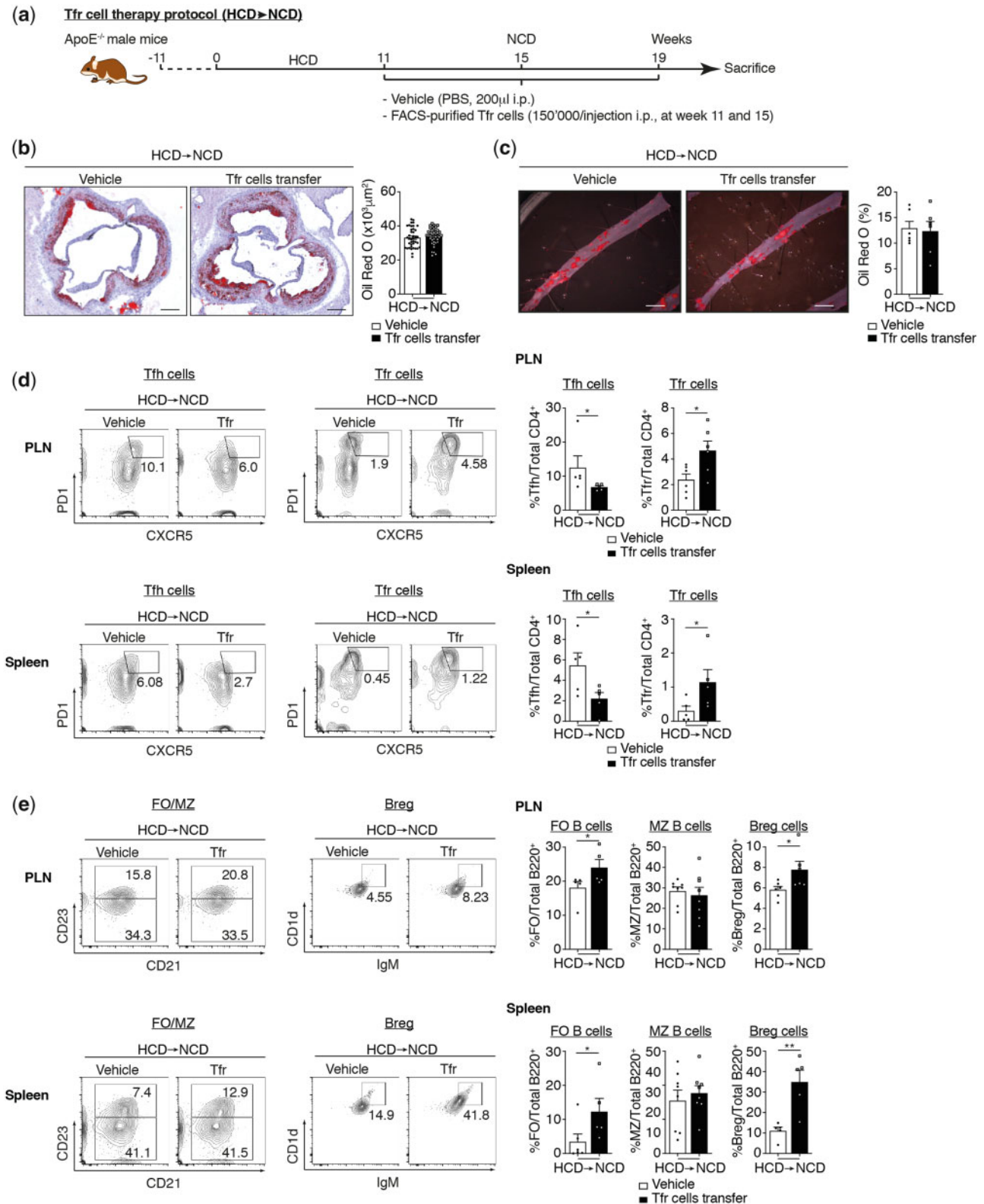


Figure 6 T_{FR} cell population is not able to repair established atherosclerosis lesions. (A) Mouse atherosclerosis protocols and adoptive transfer strategy. (B) Quantification of atherosclerotic lipid content and representative microphotographs of aortic root plaques (Oil Red O staining) in ApoE^{-/-} mice treated with vehicle (PBS) or Bcl-6 inhibitor under NCD and HCD ($n = 6$ mice/group). Original magnification, $\times 10$. Scale bars, 400 μm . (C) Quantification of atherosclerotic lipid content and representative microphotographs from Oil Red O-stained abdominal aortas ($n = 6$ mice/group). Scale bars, 500 μm . (D) Effect of T_{FR} cell transfer on secondary lymphoid organs T_{FH} and T_{FR} populations on established atherosclerosis mice ($n = 5-6$ mice/group). (E) Effect of T_{FR} cell transfer on secondary lymphoid organs in B cell populations on established atherosclerosis mice ($n = 5-8$ mice/group). The non-parametric Mann-Whitney U test was used for statistical analysis: * $P \leq 0.05$. All data were represented as mean \pm SEM.

lymphangiogenic effects of IL-4 have been established and the functions of lymphangiogenesis in atherosclerosis suggest that the lymphatic system possesses anti-atherogenic properties through the modulation of cholesterol homeostasis,^{8,14} at least in the late stages of atherosclerosis.¹² It has also been demonstrated that CD4⁺ T cells transferred to anti-CD3-treated mice decrease the development of lymph node lymphatic vessels.⁹ Additionally, Gousopoulos et al.⁴⁰ have shown that Foxp3⁺ cells transferred into mice which have undergone surgical lymphedema induction promotes lymphatic vessel function in association with a reduced level of expression of IL-4. These findings are consistent with our present data showing that T_{FR} cells stimulate lymphatic vessel expansion through the inhibition of T_{FH} cell-produced IL-4 in a HDC. Altogether, these data demonstrate that T_{FR} cell populations drive pro-lymphangiogenesis and carry anti-atherogenic functions.

The lymphatic system is intimately related to cholesterol homeostasis, and it has been suggested that Bcl-6 inhibitors are also able to modulate lipid metabolism.^{11,12,41} As a result, Bcl-6-deficient mice typically have higher serum levels of cholesterol.⁴¹ These findings are consistent with our data showing that the Bcl-6 inhibitor induces an increase in the total level of cholesterol. Inversely, it was recently shown that increasing the level of cholesterol leads to CD4⁺ T cell differentiation in Foxp3⁺ T cells.⁴² Hypercholesterolaemia thus induces Foxp3 expression and promotes T cell antigen receptor signalling in CD4⁺ T cells.⁴³ On the other hand, T_{REG} cell depletion increases atherosclerosis by modulating lipoprotein metabolism.³ Although the exact mechanism underlining the regulation of lipoprotein metabolism by regulatory immunity is not fully understood, our data indicate that the RCT-related gene *abca1*, (but not *abcg1*) is increased by Bcl6 inhibitors and decreased by adoptive transfer of T_{FR} cells through LXR α in Apoe^{-/-} mice on an HCD. While inverse variations should be expected because *abca1* and *abcg1* promote cholesterol efflux from the cells, a deficiency of *abca1* promotes foam cell accumulation and accelerates atherosclerosis in mice.⁴⁴ Similarly, enhanced *abcg1* expression increases atherosclerosis in mice with advanced atherosclerosis.^{45,46} As T_{FR} cell population is able to modulate lipid metabolism, this ability could be a part of the anti-atherogenic properties of T_{FR} cells. Overall, studies suggest that T_{REG} cell, T_{FR} cell, and lipoprotein metabolism are mutually affected, although the exact mechanisms underlying these interactions remain to be unravelled.

We further established that T_{FR} cells exert atheroprotective effects in at least three different ways. The first is by controlling expansion of the pro-atherogenic T_{FH} cell population in Apoe^{-/-} mice in advanced atherosclerosis. The second is by controlling expansion of B_{REG} cell populations in Apoe^{-/-} mice in advanced atherosclerosis and *in vitro*. The third is by controlling lymphangiogenesis through the inhibition of IL-4-dependent production by T_{FH} cell populations and decreasing lipoprotein atherogenic profiles. All of which have proved to be mostly anti-atherogenic processes.^{7,47} Finally, our results indicate that T_{FR} cell populations are unable to repair established atherosclerotic lesions. Consequently, *ex vivo* expansion of T_{FR} cell populations could be considered for cellular therapy in the context of primary, but not secondary, care treatment of atherosclerosis patients.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Authors' contributions

K.J.B. conceived, designed, and supervised the study. F.B., K.M., A.R., D.B., R.F.-S., and K.J.B. performed the experiments. K.J.B., F.B., K.M., C.M., and F.M. analysed the data. K.J.B. and F.M. wrote the article.

Acknowledgements

The authors thank also Prof. Mauri and Prof. Monaco for helpful discussions and, especially Aliko Buhayer (Prism Scientific Sàrl; www.prismscientific.ch) for critical review of the present article.

Conflict of interest: none declared.

Funding

This study was supported by European Commission (FP7-INNOVATION I HEALTH-F2-2013-602114; Athero-B-cell: targeting and exploiting B cell function for treatment in CVD). This work was also supported by Swiss National Science Foundation Grants to Prof. François Mach (No. 310030_152912/1) and a Geneva Private Foundation. The funders had no role in study design, data collection, or analysis, nor influenced the decision to publish or preparation of the article.

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Translational perspective

This research demonstrates that T_{FR} cells have atheroprotective functions through modulation of different important biological processes present during atherogenesis. Among these biological processes, lymphangiogenesis and B_{REG} proliferation appear to have particular importance across their ability to modulate immune response, inflammation, and cholesterol levels. It may be worth evaluating the use of T_{FR} cells in the primary setting for patients with familial hypercholesterolaemia, although T_{FR} cell populations cannot be considered as a means of treating established atherosclerosis.