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Fc γ RIIB Inhibits the Development of Atherosclerosis in Low-Density Lipoprotein Receptor-Deficient Mice

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The immune processes associated with atherogenesis have received considerable attention during recent years. IgG FcRs (Fc γ R) are involved in activating the immune system and in maintaining peripheral tolerance. However, the role of the inhibitory IgG receptor Fc γ RIIB in atherosclerosis has not been defined. Bone marrow cells from Fc γ RIIB-deficient mice and C57BL/6 control mice were transplanted to low-density lipoprotein receptor-deficient mice. Atherosclerosis was induced by feeding the recipient mice a high-fat diet for 8 wk and evaluated using Oil Red O staining of the descending aorta at sacrifice. The molecular mechanisms triggering atherosclerosis was studied by examining splenic B and T cells, as well as Th1 and Th2 immune responses using flow cytometry and ELISA. The atherosclerotic lesion area in the descending aorta was ~5-fold larger in mice lacking Fc γ RIIB than in control mice (2.75 ± 2.57 versus $0.44 \pm 0.42\%$; $p < 0.01$). Moreover, the Fc γ RIIB deficiency resulted in an amplified splenocyte proliferative response to Con A stimulation (proliferation index 30.26 ± 8.81 versus $2.96 \pm 0.81\%$, $p < 0.0001$) and an enhanced expression of MHC class II on the B cells (6.65 ± 0.64 versus $2.33 \pm 0.25\%$; $p < 0.001$). In accordance, an enlarged amount of CD25-positive CD4 T cells was found in the spleen (42.74 ± 4.05 versus $2.45 \pm 0.31\%$; $p < 0.0001$). The plasma Ab and cytokine pattern suggested increased Th1 and Th2 immune responses, respectively. These results show that Fc γ RIIB inhibits the development of atherosclerosis in mice. In addition, they indicate that absence of the inhibiting IgG receptor cause disease, depending on an imbalance of activating and inhibiting immune cells. *The Journal of Immunology*, 2010, 184: 2253–2260.

Accumulation and oxidation of low-density lipoprotein (LDL) particles in the arterial intima are believed to play important roles in the development of atherosclerosis (1, 2). Oxidation of these particles results in formation of oxidized phospholipids and a fragmentation of the LDL protein apolipoprotein (apo) B-100. A subsequent aldehyde modification of these apo B fragments make them targets for the immune system (3, 4). During the past decades, immune processes involved in atherogenesis have received considerable interest (5). Several lines of evidence indicate that adaptive immune responses are part of the development of atherosclerosis and promote inflammation and plaque growth (6, 7). In contrast, immunization of hypercholesterolemic animals with native or oxidized LDL have been shown to result in a significant reduction of atherosclerosis development (8, 9). In addition, existence of an atheroprotective humoral immunity is supported by studies in apo E-deficient mice demonstrating in-

hibition of atherosclerosis by repeated injections of polyclonal IgG and by B cell rescue of splenectomized mice (10, 11).

We have previously characterized a large number of different malondialdehyde-modified apo B-100 amino acid sequences specifically recognized by Abs present in human plasma (12). Immunization of apo E-deficient mice with some of these apo B-100 peptides induces an Ig switch from IgM to IgG that is accompanied by an inhibition of atherosclerosis (13–15). To study the possible atheroprotective effects of this IgG, we produced human IgG1 specific for an apo B-100 peptide. A similar inhibition of atherosclerosis together with decreased plaque macrophage immunoreactivity was observed in apo E-deficient mice following injections with the recombinant IgG1 Ab (16). The Ab treatment has also been shown to induce rapid and substantial regression of atherosclerotic lesions (17). In addition, in clinical studies, we have recently presented an independent association between high levels of IgG autoantibodies to apo B-100 peptides and a lower degree of carotid stenosis (18) and less coronary atherosclerosis as well as lower risk of myocardial infarction (19). Taken together, this may reflect that presence of IgG Abs to oxidized LDL Ags results in either protective immunity or downregulation of pre-existing, proatherogenic immune responses.

IgG Abs can bind to FcRs for IgG (Fc γ Rs) present on immune cells and regulate immune responses (20). Two general classes of Fc γ Rs are known, the activating and the inhibitory receptors, characterized by the presence of the ITAMs or ITIMs, respectively. In mice, four different classes of Fc γ Rs have been recognized: Fc γ RI, Fc γ RIIB, Fc γ RIII, and Fc γ RIV. The Fc γ RIIB is an inhibitory receptor, whereas the others are activating immune responses. The inhibitory Fc γ RIIB is important in modulating B cell activity and humoral tolerance in late stages of B cell maturation by preventing generation of autoreactive Abs (21, 22). It is a potent regulator of BCR-mediated signaling and may thereby control B cell proliferation, class switching, and plasma cell maturation. Fc γ RIIB is

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Abbreviations used in this paper: apo, apolipoprotein; BM, bone marrow; BMT, bone marrow transplantation; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; MHC II, MHC class II; WT, wild-type.

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the only FcγR on B cells, but it is coexpressed on virtually all leukocytes together with the activating FcγRs, except for NK and T cells. Alterations in the expression ratio of activating/inhibitory FcγR could impair regulated priming of Ag-specific T cells (22). In line with this, it has been shown that FcγRIIB-deficient mice on a C57BL/6 background develop a lupus-like disease and high titers of autoantibodies, indicating that loss of the negative regulator leads to imbalanced immune responses resulting in autoimmunity (23, 24).

Previous experimental studies have presented an involvement of FcγR activation in inflammatory and immune diseases (21, 25). The role of FcγRs in the development of atherosclerosis, however, has not been extensively evaluated. In clinical studies, FcγRs have been detected in human atherosclerotic lesions, and the expression of FcγRIIA on peripheral blood monocytes has been found to be decreased in patients with severe atherosclerosis (26, 27). In addition, it has been reported that mice deficient in the Fcγ-chain and apo E have a limited development and progression of high-fat diet-induced atherosclerosis (28). One explanation might be that deficiency of the activating FcγRs results in protection against atherosclerosis. However, this mouse model still expresses the inhibitory receptor FcγRIIB, and the presence of this receptor on the leukocytes could provide an alternative explanation for the atheroprotection. In the current study, the purpose was to evaluate the role of FcγRIIB in atherosclerosis.

Materials and Methods

Mice

Female FcγRIIB-deficient mice on a C57BL/6 background were purchased from Taconic Farms (Ry, Denmark). Female C57BL/6 wild-type (WT) and LDL receptor (LDLR)-deficient mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). Food and tap water were administered ad libitum. All animals were maintained under specific pathogen-free conditions at Lund University. This study was reviewed and approved by the local Animal Care and Use Committee.

Bone marrow transplantations

Twelve-week-old female LDLR deficient mice were used as recipient mice, subjected to 1×900 rad irradiation and randomly assigned to receive bone marrow (BM) from 9- to 11-wk-old FcγRIIB-deficient or C57BL/6 mice. BM was harvested by flushing the femurs and tibias of the donor mice, RBCs were lysed in 0.2% NaCl at room temperature for 5 min, and BM cells were washed and resuspended in PBS. Recipient mice were injected with $0.2 \text{ ml } 2 \times 10^6$ BM cells through the tail vein. One day before and for a duration of 7 d after the BM transplantation, recipient mice were given 2 mg/ml neomycin sulfate (Sigma-Aldrich, St. Louis, MO) in the drinking water. Confirmation of transplantation was performed with PCR analysis of blood cells, lysed in $10 \text{ mmol/l Tris-HCl}$ (pH 8.0) and 1% Tween 20, and digested with $400 \text{ } \mu\text{g/ml}$ proteinase K at 60°C for 2 h. Primers specific for the LDLR of the donor mice (oIMR0092 [Mut], 5'-AAT CCA TCT TgT TCA ATg gCC gAT C-3'; oIMR3349 [common], 5'-CCA TAT gCA TCC CCA gTC TT-3'; and oIMR3350 [WT], 5'-gCg ATg gAT ACA CTC ACT gC-3') were used in the PCR analysis. Four weeks after transplantation, mice were given a high-fat diet (0.15% cholesterol and 21% fat; Lantmännens, Stockholm, Sweden) for another 8 wk. Mice were sacrificed by i.p. injection of ketamine and xylazine.

Tissue preparation

Spleens were harvested and stored in PBS on ice. Plasma was collected by cardiac puncture and stored at -80°C until analyzed. After whole-body perfusion with PBS followed by Histochoice (Amresco, Solon, OH), the descending aorta was dissected free of connective tissue and fat, cut longitudinally, mounted en face, and stored in Histochoice (16).

Staining of the descending aorta

En face preparations of the descending aorta were washed in distilled water, dipped in 78% methanol, and stained for 40 min in 0.16% Oil-Red-O dissolved in 78% methanol/0.2 mol/l NaOH (29). The stained plaque areas were quantified blindly using Image pro plus 4.5 software (Media Cybernetics, Bethesda, MD).

Spleen cell preparation and cultures

Splenocytes in single cell suspension were prepared by pressing spleens through a $70\text{-}\mu\text{m}$ cell strainer (BD Falcon, Franklin Lakes, NJ). Erythrocytes were removed using RBC lysing buffer (Sigma-Aldrich). Cells were cultured in culture media (RPMI 1640 medium containing 10% heat-inactivated FCS, 1 mmol/l sodium pyruvate, 10 mmol/l HEPES, 50 U penicillin, $50 \text{ } \mu\text{g/ml}$ streptomycin, 0.05 mmol/l β -mercaptoethanol, and 2 mmol/l L-glutamine; Invitrogen Life Technologies, Carlsbad, CA) in 96-well round bottom plates (Sarstedt, Landskrona, Sweden). In proliferation assays, 2×10^5 splenocytes/well were cultured alone or with $2.5 \text{ } \mu\text{g/ml}$ Con A (Sigma-Aldrich) for 90 h. To measure DNA synthesis, the cells were pulsed with $1 \text{ } \mu\text{Ci}$ [methyl- ^3H]thymidine (Amersham Biosciences, Uppsala, Sweden) during the last 16 h. Macromolecular material was then harvested on glass fiber filters using a Printed Filtermat A (1450-421; Wallac Oy, Turku, Finland). Filters were air-dried, and the bound radioactivity was measured in a beta counter (Wallac 1450; MicroBeta, Ramsey, MN). The result is shown as a proliferation index (Con A-stimulated cells divided with nonstimulated). In parallel, 3×10^5 splenocytes/well were cultured alone or with $2.5 \text{ } \mu\text{g/ml}$ Con A for 72 h, and cytokine concentrations were measured in the cell culture supernatant using a Th1/Th2 9-plex Ultra-Sensitive Kit (Meso Scale Discovery, Gaithersburg, MO) according to the instructions of the manufacturer. The lower detection limit for all cytokines in this assay is $\sim 1.0 \text{ pg/ml}$.

Flow cytometry

Splenocytes were stained with fluorochrome-conjugated Abs and analyzed with a CyAn ADP flow cytometer (Beckman Coulter, Fullerton, CA) and the Summit software (DakoCytomation, Fort Collins, CO). The Abs used in these experiments were recognizing CD4, CD8, CD25, B220, HLA-DR (all from Biolegend, San Diego, CA), or Foxp3 (eBioscience, San Diego, CA). The Foxp3 intracellular staining followed the recommended instructions of the manufacturer.

Plasma-oxidized LDL-specific Abs and apo B immune complex analysis

Plasma-oxidized LDL-specific Abs were analyzed in Cu^{2+} -oxidized LDL ($10 \text{ } \mu\text{g/well}$ in PBS)-coated microtiter plates (30). The plasma Abs were detected using Abs recognizing mouse IgG (Abcam, Cambridge, MA), IgM (ICN Pharmaceuticals, Aurora, OH), IgG1 (557272; BD Pharmingen, San Jose, CA), and IgG2a/c (553389; BD Pharmingen). To measure the levels of total IgG1 and IgG2a/c in plasma, the assays were performed using the capture Abs (IgG1: 553445 and IgG2a/c: 553446; BD Pharmingen) as suggested by the manufacturer in combination with the detection Abs described above. In the apo B immune complex assay, microtiter plates were coated with rabbit anti-apo B Ab (Abcam), and the plasma apo B containing immune complexes were detected with the anti-mouse IgG1 and IgG2a/c detection Abs described above.

Plasma cholesterol, triglyceride, cytokine, and chemokine analysis

Total plasma cholesterol and plasma triglycerides were quantified with colorimetric assays, infinity cholesterol, and triglyceride (Thermo Scientific, Liverpool, U.K.). Plasma cytokine concentrations were analyzed using a Meso Scale Discovery mouse Th1/Th2 9-Plex (IFN- γ , IL-1 β , TNF- α , IL-2, IL-12, IL-4, IL-5, IL-10, and KC) Ultra-Sensitive Kit (Meso Scale Discovery), following the instructions of the manufacturer. The presence of the chemokines CCL2 and CCL5 in plasma were measured using DuoSet ELISAs (CCL2/IE product number DY479 and CCL5/RANTES product number DY478; R&D Systems, Minneapolis, MN).

Statistics

Data are presented as mean $\pm$ SD. Statistical significance was determined using the nonparametric Mann-Whitney *U* test. Statistical significance was considered at the level $p \leq 0.05$.

Results

FcγRIIB deficiency increase the atherosclerosis development

To study the role of the FcγRIIB in the atherosclerosis development, BM cells from C57BL/6 (WT) or FcγRIIB-deficient mice were transplanted to LDLR-deficient mice. The design of the experiment is outlined in Table I. Presence of the LDLR in blood cells of LDLR-deficient recipient mice, tested by PCR, confirmed

Table I. Experimental design

Donor (D)	Recipient (R)	No. of Mice (D/R)	BM Transfer (wk)	Diet (wk)	Killing (wk)
C57BL/6	LDLR ^{-/-}	10/10	12	16	24
FcγRIIB ^{-/-}	LDLR ^{-/-}	10/10	12	16	24

-/-, deficiency; C57BL/6, WT control mice; diet, high-fat diet; wk, weeks of age.

a successful transplantation (data not shown). Four weeks after the BM transplantation, mice were given high-fat diet for 8 wk to induce atherosclerosis development. At sacrifice the descending aorta was taken out and stained with Oil Red O to visualize the lipid containing atherosclerotic plaques. A significantly increased plaque area was observed in mice transplanted with FcγRIIB-deficient BM cells compared with mice transplanted with WT cells (2.75 ± 2.57 versus $0.44 \pm 0.42\%$; $p < 0.01$) (Fig. 1). Three mice in the FcγRIIB-deficient BM cell recipient group were found to have more severe atherosclerosis than the others. However, we found no differences in any of the immunological parameters tested that could explain the variation. There were also no differences in plasma cholesterol or triglyceride levels between the groups (Table II).

Increased proliferation of splenocytes in mice transplanted with FcγRIIB-deficient BM cells

In the initiation of the atherosclerosis development, oxidized LDL particles play a key role. During the past decades, it has been more and more obvious that the modified lipoprotein particle becomes a target for the immune system and thereby enhances the disease process (31). To evaluate the activation of the immune system in the absence of FcγRIIB, splenocytes from the BM recipient mice were stimulated with Con A, and the proliferation was examined. Con A-induced splenocyte proliferation was found to be increased

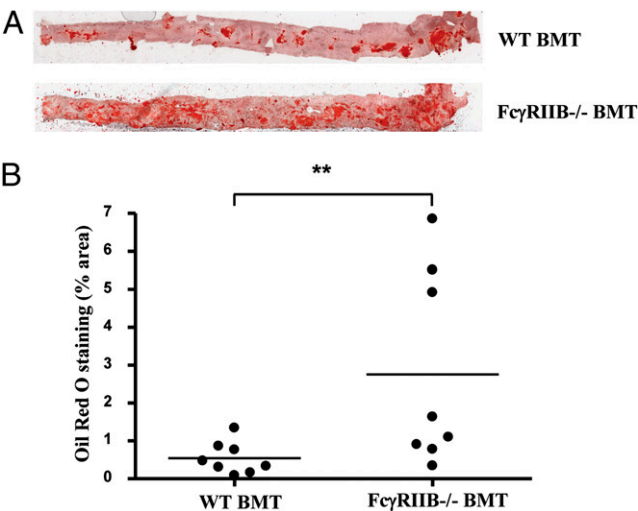


FIGURE 1. FcγRIIB deficiency in LDLR-deficient mice induces more atherosclerosis. Twelve-week-old LDLR-deficient mice received BM cells from C57BL/6 (WT) or FcγRIIB-deficient mice. Four weeks after the transplantation, the diet was changed to a high-fat diet. The mice were killed at the age of 24 wk, and the aorta was dissected free. *A*, Representative images of Oil Red O stained descending aortas of mice transplanted with BM cells from a C57BL/6 (WT) or FcγRIIB-deficient mouse, respectively, are shown. *B*, Plaque areas in descending aortas were assessed by the en face Oil Red O staining, and the percent stained area of total aortic area was determined by computerized image analysis. ** $p < 0.01$. BMT, BM transplantation.

Table II. Plasma cholesterol and triglyceride

BM Donor Mice	Cholesterol (mg/dl)	Triglyceride (mg/dl)
C57BL/6 (WT)	915.4 ± 306.5	457.6 ± 164.4
FcγRIIB ^{-/-}	878.4 ± 139.9	401.9 ± 79.7

-/-, deficiency; $n = 10$ /group.

severalfold in mice transplanted with FcγRIIB-deficient BM cells compared with mice transplanted with WT cells (proliferation index 30.26 ± 8.81 versus 2.96 ± 0.81 ; $p < 0.0001$) (Fig. 2).

More B cells in the spleen of mice transplanted with FcγRIIB-deficient BM cells

Depletion of the inhibitory FcR for IgG might induce an enhanced B cell activity and modulate the humoral tolerance in late stages of B cell maturation (21, 22). In accordance, we found that the portion of B cells in the spleen was significantly increased in mice transplanted with FcγRIIB-deficient BM cells compared with mice transplanted with WT cells (55.21 ± 10.93 versus $31.60 \pm 9.01\%$; $p < 0.001$) (Fig. 3*B*). Furthermore, the Ag-presenting MHC class II (MHC II) molecule showed a higher expression on B cells in the absence of FcγRIIB (6.65 ± 0.64 versus $2.33 \pm 0.25\%$; $p < 0.001$) (Fig. 3*A*, 3*C*).

More activated CD4⁺ T cells in mice transplanted with FcγRIIB-deficient BM cells

To further characterize the effect of FcγRIIB deficiency, we analyzed the T cell population in the spleen. The analysis of the CD4⁺ and CD8⁺ T cell populations showed a significantly smaller CD8⁺ T cell population in mice transplanted with FcγRIIB-deficient BM cells than mice transplanted with WT cells (4.88 ± 1.04 versus $7.22 \pm 2.31\%$; $p < 0.05$), whereas the CD4⁺ T cells were in the same range (16.57 ± 1.46 versus $14.70 \pm 4.37\%$; ns). This resulted in a higher CD4⁺/CD8⁺ ratio in mice transplanted with FcγRIIB-deficient BM cells compared with mice transplanted with WT cells (3.53 ± 0.24 versus $1.95 \pm 0.11\%$; $p < 0.0001$). The deficiency was also associated with a dramatic increase of the activation marker CD25 on CD4⁺ T cells in the spleen (42.74 ± 4.05 versus $2.45 \pm 0.31\%$; $p < 0.001$) (Fig. 4*B*). Most of these CD4⁺CD25⁺ T cells present in mice transplanted with FcγRIIB-deficient BM cells appeared to be effector cells because the percentage of cells also expressing FoxP3 was markedly lower than in mice transplanted with WT BM cells (27.57 ± 13.77 versus $80.29 \pm 6.65\%$; $p < 0.0001$) (Fig. 4*C*). This indicates that FcγRIIB deficiency increase the activated CD4⁺ T cell population causing an imbalance between activated T effector and regulatory cells.

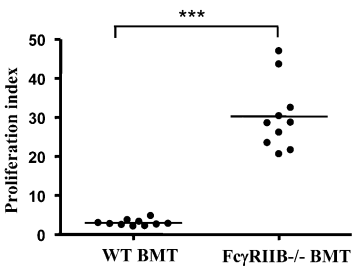
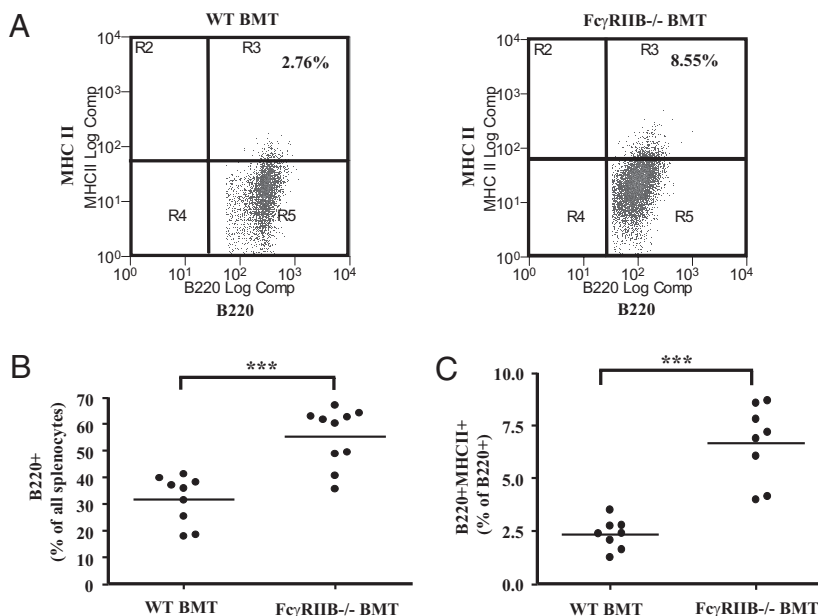


FIGURE 2. FcγRIIB deficiency induces a higher splenocyte proliferation rate. Cultured splenocytes from BM-transplanted, LDLR-deficient mice were stimulated with Con A for 90 h in the presence of radioactive-labeled thymidine incorporation during the last 16 h. T cell proliferation index is expressed as thymidine incorporation ratio between stimulated and nonstimulated cells. *** $p < 0.0001$.

FIGURE 3. FcγRIIB deficiency increases the fraction of splenic B cells. Splenocytes of LDLR-deficient mice with C57BL/6 (WT) or FcγRIIB-deficient BM cells were stained with fluorochrome-conjugated Abs recognizing B220 or HLA-DR (MHC II) and analyzed with a CyAn ADP flow cytometer. A, Splenocytes were first gated for CD11c⁻ and B220⁺ cells to obtain an enriched B cell population and thereafter for the B220⁺ cells expressing MHC II. Graphs showing the cell populations from one representative mouse from each group. The percentages given are out of the total splenocyte population (B) and B220⁺ cells (C), respectively. ****p* < 0.001. BMT, BM transplantation.



The cytokine profile in plasma and cell supernatants of mice transplanted with FcγRIIB deficient BM cells indicates a Th2 immune response

To further evaluate the influence of FcγRIIB on the T cell population, the balance of Th1/Th2 immune response was studied by measuring cytokine levels in plasma and cell supernatants of nonstimulated and Con A-stimulated splenocytes. The plasma cytokine profile showed a significant increase of IL-4 (10.93 ± 3.45 versus 7.63 ± 3.63 pg/ml; *p* < 0.05), IL-5 (27.15 ± 6.41 versus 18.08 ± 4.14 pg/ml; *p* < 0.01), and IL-10 (287.8 ± 73.0 versus 156.3 ± 56.5 pg/ml; *p* < 0.001) in mice transplanted with FcγRIIB-deficient BM cells compared with mice transplanted with WT cells, indicating activation of a Th2 immune response (Fig. 5). The other plasma cytokines tested (IFN-γ, IL-1β, IL-2, IL-12, and TNF-α) were not affected by FcγRIIB deficiency (data not shown). In analysis of the chemokine profile in the plasma, there was a trend against higher levels of CCL2 (JE) in mice transplanted with FcγRIIB-deficient BM cells (10.77 ± 4.63 versus 6.55 ± 3.78 in mice transplanted with WT cells; *p* = 0.052)

and significantly lower CXCL1 (KC) levels (163.3 ± 58.6 versus 291.1 ± 185.4 in mice transplanted with WT cells; *p* < 0.05), whereas CCL5 (RANTES) was below the detection limit in both groups. In the cell supernatants of Con A-stimulated cultured splenocytes, IL-4, IL-5, and IL-10 release were significantly increased, and IL-12 release significantly reduced in cells from mice transplanted with FcγRIIB-deficient BM cells, compatible with an expansion of the Th2 cell population (Fig. 6B). In contrast, Con A-induced IL-2 was increased, whereas there was no differences in IFN-γ, IL-1β, TNF-α, and CXCL1 release between the groups. Splenocytes from mice transplanted with FcγRIIB-deficient BM cells were also characterized by a lower basal expression of IFN-γ and IL-2, whereas that of IL-12 was increased (Fig. 6A). Notably, stimulation of these cells with Con A was found to result in an inhibition of IL-12 release (377.4 ± 77.4 versus 159.8 ± 77.6 ; *p* < 0.0001) and restored IFN-γ production (2635 ± 1331 versus 3014 ± 551 ; ns) (Fig. 6B). The basal expression of IL-10, IL-1β, TNF-α, and CXCL1 were unaffected of the deficiency (IL-4 and IL-5 levels were below detection limit).

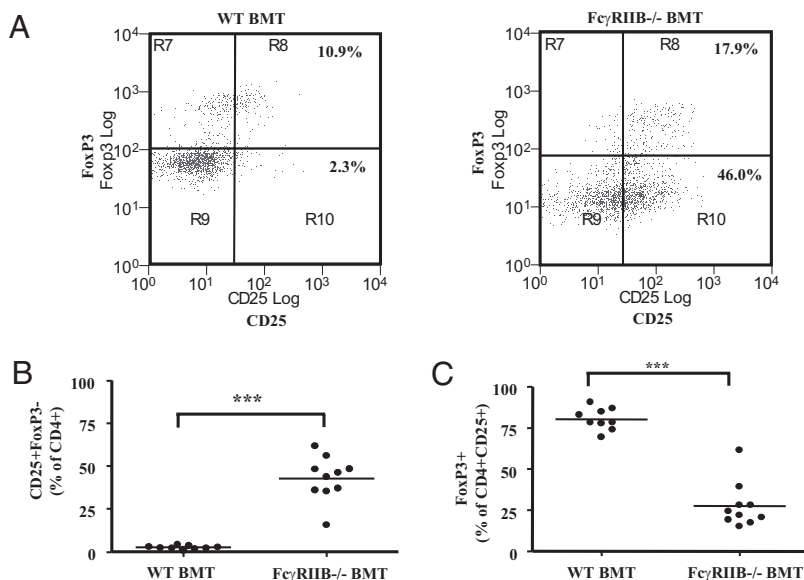


FIGURE 4. FcγRIIB deficiency influences CD4⁺ T cells activation. Splenocytes from LDLR-deficient mice with C57BL/6 (WT) or FcγRIIB-deficient BM cells were stained with fluorochrome-conjugated Abs recognizing CD4, CD25, or FoxP3 and analyzed with a CyAn ADP flow cytometer. A, Graphs showing the cell populations from one representative mouse from each group. The percentages given are the CD25⁺FoxP3⁺ cells (B) out of total CD4⁺ T cells or the FoxP3⁺ cells (C) out of the CD4⁺CD25⁺ T cell population, respectively. ****p* < 0.001. BMT, BM transplantation.

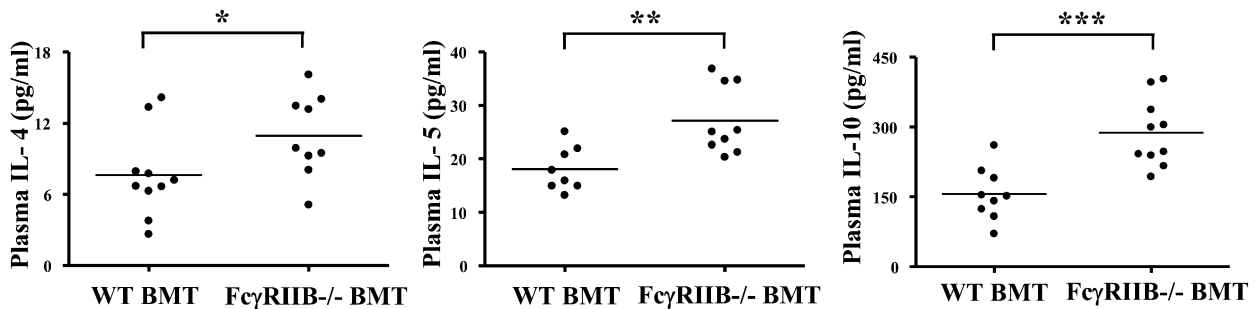


FIGURE 5. FcγRIIB deficiency induces plasma Th2 cytokines. Plasma cytokine levels in LDLR-deficient mice with C57BL/6 (WT) or FcγRIIB-deficient BM cells were analyzed by multiplex technology (Meso Scale Discovery). IL-4, IL-5, and IL-10 plasma levels were significantly increased in absence of FcγRIIB. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. BMT, BM transplantation.

The plasma Ab pattern in mice transplanted with FcγRIIB-deficient BM cells indicates Th1 immunity

It has previously been shown that FcγRIIB-deficient mice on a C57BL/6 background have high titers of autoantibodies (23). Moreover, mice with moderate hypercholesterolemia have been found to have elevated levels of autoantibodies to oxidized LDL, largely of the IgG2a isotype (30). In the current study, the hypercholesterolemic mice lacking FcγRIIB had significantly higher levels of oxidized LDL-specific IgG Abs, whereas no difference was found in IgM Abs (Fig. 7A, 7B). Furthermore, the oxidized LDL IgG2a/c Abs were elevated severalfold, whereas the IgG1 Abs showed only a minor nonsignificant increase (Fig. 7C, 7D), indicating a more pronounced Th1 immune response. In accordance, total IgG2a/c Abs were significantly increased (0.70 ± 0.07 versus 0.26 ± 0.03 absorbance units; $p < 0.0001$), and no difference was detected in total IgG1 plasma levels (1.02 ± 0.02 versus 1.06 ± 0.02 absorbance units; $p = 0.25$). To measure plasma LDL containing immune complexes, an Ab against the apo B was used in combination with Abs recognizing either IgG1 or

IgG2a/c in a sandwich ELISA. Immune complexes, including IgG2a/c Abs, were markedly increased in mice transplanted with FcγRIIB-deficient BM cells, whereas the IgG1 containing immune complexes showed only a small elevation (Fig. 8A, 8B). Altogether, the Ab pattern suggests a more pronounced Th1 immune response.

Discussion

In the current study, we observed that absence of FcγRIIB induces increased atherosclerosis development in LDLR-deficient mice. Absence of FcγRIIB was also associated with an expansion and increased activation of B cells, an increased activation of CD4-positive T cells, and an impaired balance between T effector and regulatory cells. In accordance, the splenocytes showed a higher proliferation rate. The cytokine profile in the blood, as well as in the cell supernatants of Con A-stimulated cultured splenocytes, indicated an increased Th2 immune response. In contrast, plasma Abs recognizing oxidized LDL, as well as apo B containing immune complexes, revealed an increase of Th1 specific IgG2a/c Abs. Accordingly, in the current study, mice transplanted with FcγRIIB BM cells demonstrates signs of activation of both Th1 and Th2 immunity.

The development of atherosclerosis has been extensively studied in mouse models deficient in apo E or the LDLR, because both animal models develop complex atherosclerotic lesions that are

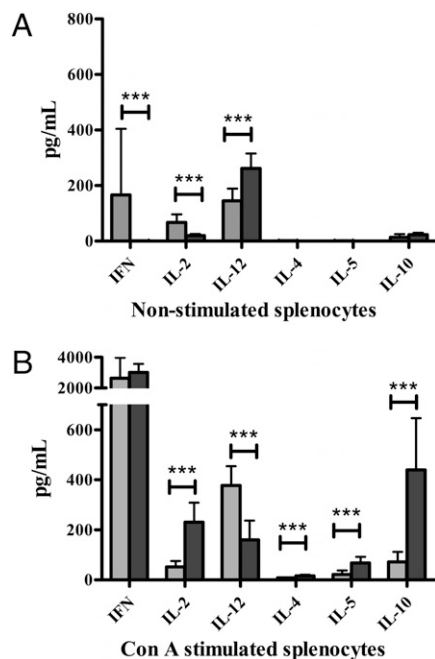


FIGURE 6. FcγRIIB deficiency induces primarily a Th2 cytokine profile in the spleen. Cytokine levels in supernatants of nonstimulated (A) or Con A-stimulated (B) cultured spleen cells from LDLR-deficient mice with C57BL/6 (WT, light gray bars) or FcγRIIB-deficient (dark gray bars) BM cells were analyzed by multiplex technology (Meso Scale Discovery). *** $p < 0.001$. IFN, IFN- γ .

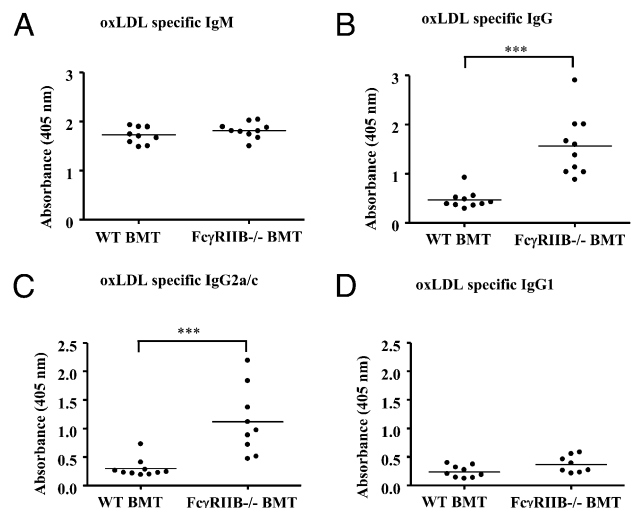


FIGURE 7. FcγRIIB deficiency induces Th1 autoantibody immunity. Plasma from LDLR-deficient mice with C57BL/6 (WT) or FcγRIIB-deficient BM cells were analyzed for oxidized LDL-specific IgM (A), IgG (B), IgG2a/c (C), and IgG1 (D) autoantibodies. Deficiency in FcγRIIB resulted in significantly increased levels of IgG and IgG2a/c. *** $p < 0.001$. BMT, BM transplantation.

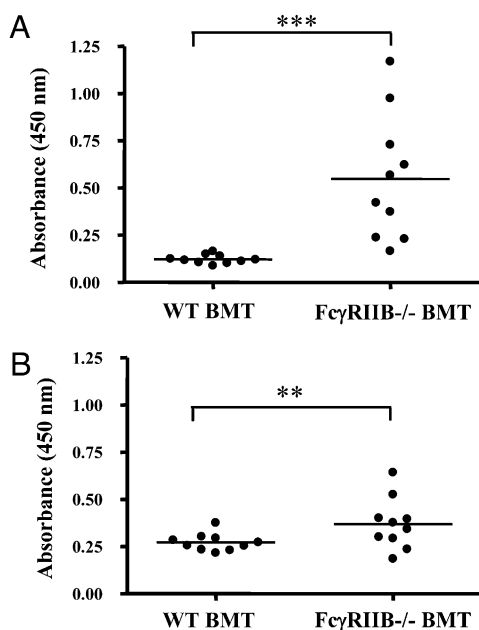


FIGURE 8. FcγRIIB deficiency increases apo B containing immune complexes in plasma. Plasma from LDLR-deficient mice with C57BL/6 (WT) or FcγRIIB-deficient BM cells were analyzed for apo B containing immune complexes. In absence of FcγRIIB, a 3-fold elevated level of apo B/IgG2a/c immune complexes was detected (A), together with a small increase in apo B/IgG1 immune complexes (B). ** $p < 0.01$; *** $p < 0.001$. BMT, BM transplantation.

similar to the plaque formation in humans (32). A previous study focused on the role of the FcγR in atherosclerosis by using a double-knockout mice deficient in apo E and the FcγRγ-chain (28). This mouse model developed ~50% less atherosclerotic lesions in the aorta and showed reduced expression of the MCP-1 and RANTES in the aortic lesions. Taken together, this indicates that FcγR deficiency limits the development and progression of atherosclerosis. However, this mouse model was shown to have an enhanced expression of the inhibitory FcγRIIB receptor that potentially could be responsible for the observed atheroprotection. Thus, the current study investigated the possibility that FcγRIIB limits the atherosclerosis development in LDLR-deficient mice. There is abundant evidence that proinflammatory Th1 immune responses against altered self-Ags generated by hypercholesterolemia, such as oxidized LDL particles, have a predominance in atherosclerosis (31, 33–37). Both innate and acquired proatherogenic immunity is activated by hypercholesterolemia, supporting the concept that the disease at least in part should be considered as an autoimmune disease (5, 6). The absence of FcγRIIB may pronounce the proinflammatory Th1 immune response and aggravate the disease, because it has been shown that the receptor contributes to maintenance of tolerance and protection from autoimmune disease (22, 23). Accordingly, our observations suggest the possibility that FcγRIIB helps to control autoimmune responses against modified self-Ags generated by hypercholesterolemia and through this mechanism protects against the development of atherosclerosis.

B cells are less common in atherosclerotic plaques in comparison with macrophages and T cells (38). However, atherosclerosis seems to be associated with production of autoantibodies recognizing Ags in the oxidized LDL particle (12, 39, 40). In contrast, an atheroprotective immunization of apo E-deficient mice with apo B-100 peptides resulted in increased levels of specific IgG Abs and a change in the immune response from IgG2a/c to IgG1

(14). Furthermore, clinical studies have revealed that high levels of IgG to native apo B-100 peptides are associated with lower risk of cardiovascular disease, whereas high Ab levels against the corresponding malondialdehyde-modified peptides are associated with more severe disease (12, 19, 41). These findings may reflect that autoantibodies against mildly modified LDL particles are more beneficial than those recognizing severely modified forms of LDL. The importance of the inhibitory FcγRIIB in modulating B cell activity and humoral tolerance is supported by studies in both mice and humans (42–44). In line with these studies, we observed increased amount of splenic B cells and higher levels of plasma autoantibodies recognizing oxidized LDL in the absence of FcγRIIB. Moreover, the B cells showed a higher expression of the Ag-presenting molecule MHC II on the surface, indicating an enhanced activation. Previously, the inhibitory receptor has also been shown to control the magnitude of Ag-presenting responses, suggesting that absence of the receptor generates stronger and longer-lasting immune responses (45). The Ab pattern, showing higher levels of predominantly oxidized LDL-specific IgG2a/c autoantibodies, as well as increased levels of total IgG2a/c, in our hypercholesterolemic mouse model transplanted with FcγRIIB-deficient BM cells, is well in agreement with the hypothesis that atherosclerosis is driven by Th1 immune responses. Furthermore, the elevated levels of autoantibodies reflect findings in previous studies reporting that FcγRIIB controls BM plasma cell persistence and that absence of the receptor results in autoantibody production and unsatisfactory follicular exclusion of autoreactive B cells (43, 44).

Several studies have demonstrated an atheroprotective role of regulatory T cells. Deficiency in ICOS, TGF-β, or IL-10 in atherosclerosis prone mice demonstrates acceleration of the disease development (46–48). Moreover, regulatory T cells from apo E-deficient mice show reduced functional suppressive properties compared with those from WT mice (49). The role of FcγRIIB in regulation of T cells has not been extensively studied. Bolland and Ravetch (23) have previously reported an increase in CD4⁺ cells relative to CD8⁺ cells in the spleen and an elevated expression of the activation marker CD69 on splenic CD4 cells in FcγRIIB-deficient mice on a C57BL/6 background. Another study reported lower expression of FoxP3 on CD4⁺ cells in the inguinal lymph nodes in FcγRIIB-deficient mice compared with the control C57BL/6 mice and a higher incidence of experimental autoimmune encephalomyelitis (50). The findings in the current study are in accordance with the results presented in the two latter studies. Thus, the higher amount of activated splenic T cells may contribute to the increased atherosclerosis development detected in our mice. A potential role of Th17/regulatory T cell imbalance for atherosclerosis development in apo E-deficient mice has been described previously (51). It has to be elucidated whether Th17 cells also have a role in atherogenesis in our hypercholesterolemic mouse model transplanted with FcγRIIB-deficient BM cells. Surprisingly, the deficiency resulted in higher levels of the anti-inflammatory cytokines IL-4, IL-5, and IL-10. Contrarily, FcγRIIB deficiency generated in a mouse strain permissive for autoimmune disease greatly increased IL-12 expression (24), but the absence of FcγRγ-chain and apo E showed reduced expression of MCP-1 and RANTES in aortic lesions (28). Interestingly, in the current study, the FcγRIIB-deficient mice were fed an atherogenic diet that might result in a stronger Th1 reaction and a more severe disease and thereby the immune system may attempt to compensate and balance the response by increased production of anti-inflammatory cytokines.

In conclusion, our data provide evidence that FcγRIIB limits the atherosclerosis development in hypercholesterolemic mice.

Furthermore, they indicate that deficiency of the inhibitory receptor may promote disease by an imbalance between regulatory and pathogenic immunity. Additionally, the study gives new insights to the involvement of immune mechanisms in atherosclerosis development.

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Disclosures

The authors have no financial conflicts of interest.

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