Crosstalk between apoptosis and inflammation in atherosclerosis

Westra, M.M.

Chapter 4

Leukocyte Bim Deficiency Induces Anti-Ox-LDL Autoantibody Formation and T Cell and Immunoglobulin Accumulation in Atherosclerotic Lesions of LDLr$^{-/-}$ Mice

Marijke M. Westra[1], Ilze Bot[1], Martine Bot[1], Kim L.L. Habets[1], Saskia C.A. de Jager[1], Tom G.H. Keulers[1], Thomas G. Cotter[2], Theo J.C van Berkel[1] and Erik A.L. Biessen[1,3]

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[1] Division of Biopharmaceutics, Leiden Amsterdam Centre for Drug Research, Leiden University, Leiden, The Netherlands
[2] Cell Development and Disease Laboratory, Department of Biochemistry, Biosciences Research Institute, University College Cork, Cork, Ireland
[3] Experimental Vascular Pathology group, Department of Pathology, Maastricht University Medical Center, Maastricht, The Netherlands

Abstract

Proapoptotic Bcl-2 family member Bim is an important regulator of leukocyte apoptosis. It is particularly relevant for the deletion of autoreactive and activated T and B cells implicating Bim in autoimmunity. As atherosclerosis is regarded as a chronic inflammatory process with features of an autoimmune disease, we investigated the impact of hematopoietic Bim deficiency on plaque formation in Western type diet fed LDLr$^{-/-}$ mice.

Bim$^{-/-}$ bone marrow transplanted LDLr$^{-/-}$ mice displayed marked splenomegaly. The pro-inflammatory status of Bim$^{-/-}$ chimeras was also reflected by enhanced T cell activation and proliferation, which could be partly attributed to an increased capacity of matured Bim$^{-/-}$ DCs to induce T cell proliferation. Moreover, circulating and mediastinal lymph node lymphocyte levels were increased while atherosclerotic lesions were seen to be enriched in T cells as well. Leukocyte Bim deficiency was accompanied by elevated levels of Ox-LDL specific autoantibodies of the IgG1 but not IgG2a isotype, suggestive of a Th2 biased T-cell response. In addition, plaques of Bim$^{-/-}$ but not WT transplanted mice contained massive immunoglobulin deposits. Although circulating monocyte levels were increased in Bim$^{-/-}$ BM recipients and bone marrow derived macrophages proved to be less sensitive for Ox-LDL induced apoptosis, Bim deficiency did not translate in altered lesion macrophage content. Surprisingly, despite these profound effects and unexpected 30-50% reductions in serum lipid levels, atherosclerotic lesion burden in aortic root and descending aorta as well as lesion stability were unaffected in Bim$^{-/-}$ BM transplanted mice.

In conclusion, leukocyte Bim deficiency in LDLr$^{-/-}$ mice results in general T cell activation, increased lesion T cell but not macrophage content, enhanced anti-Ox-LDL autoantibody formation and lesional immunoglobulin deposition. Apparently, pro-inflammatory effects counteract the atheroprotective effect of Bim deficiency on serum lipid levels leading to an overall unchanged atherogenic response.

Introduction

Atherosclerosis is a chronic inflammatory disease characterized by lipid accumulation[1]. With disease progression vascular apoptosis gradually increases[2]. In early atherogenesis apoptosis is regarded a beneficial factor as it dampens plaque inflammation and limits lesion expansion[3,4]. At later stages of atherosclerosis however clearance of apoptotic cells has been demonstrated to be insufficient[5], and apoptotic cell death might here promote necrotic core formation and inflammation.

The Bcl-2 family of pro- and antiapoptotic proteins regulates apoptosis induced by cellular stressors such as DNA damage, UV radiation and oxidative stress[6]. Proteins of this family share one to four Bcl-2 homology (BH) domains[6]. *In vivo* studies in atherosclerotic mouse models have established a role for various Bcl-2 family members in apoptosis of atherosclerotic lesion macrophages[7,8]. For instance lesions of lethally irradiated LDLr$^{-/-}$ mice transplanted with proapoptotic Bax deficient bone marrow were seen to contain 53% less apoptotic cells and demonstrated to be 50% larger than Bax$^{+/+}$ transplanted mice[7]. However in ApoE$^{-/-}$ mice macrophage specific deletion of antiapoptotic Bcl-2 did not result in altered lesion size, despite an increase in apoptotic cell content[8]. Bim (Bcl-2 interacting mediator of cell death) is a BH3-only proapoptotic protein of the Bcl-2 family[9,10] and, like other BH3-only proteins, binds to antiapoptotic Bcl-2 family members thereby initiating apoptosis[11]. Studies in Bim deficient mice have revealed crucial functions in leukocyte homeostasis. Bim deficient granulocytes and lymphocytes are less sensitive to apoptosis induced by cytokine deprivation or various apoptotic stimuli[12,13]. Moreover, Bim deficient mice have markedly increased numbers of B and T cells in circulation, spleen and thymus, and of circulating monocytes and granulocytes[12]. Bim deficiency results in auto-immunity and lymphadenopathy due to defective removal of autoreactive T and B cells[12,14,15]. Bim was also demonstrated to be necessary for appropriate termination of immune responses[16].

Atherosclerotic lesions contain a large number of inflammatory cell types including lymphocytes. Lymphocyte deficiency in mice has previously been demonstrated to result in reduced atherosclerotic lesion size[17,18] while transfer of CD4^{+} T cells in immunodeficient mice was seen to aggravate atherosclerosis[19]. Various mediators of both innate and adaptive immunity are involved in the pathogenesis of atherosclerosis. In fact atherosclerosis exhibits several features of an autoimmune disease[20-22], at which Ox-LDL derived epitopes and heat shock proteins (HSP) were shown to act as autoantigen[22]. T-cell autoreactivity is generally suppressed by regulatory T cells (Tregs), and Treg deficiency can result in autoimmune disease[23]. Concordant with various autoimmune diseases, also in atherosclerosis Treg function is impaired[23]. Likewise functional studies demonstrated that depletion or functional impairment of Tregs exacerbated atherosclerosis in ApoE$^{-/-}$ mice[24,25]. As Bim is instrumental in preventing autoimmunity a role for this protein in the pathogenesis of atherosclerosis is anticipated. Therefore, in the present study we have investigated the role of Bim regulated apoptosis in atherosclerosis-prone LDLr$^{-/-}$ mice. Our study shows that leukocyte Bim deficiency in LDLr$^{-/-}$ mice results in increased atherosclerotic

lesion T cell content and massive immunoglobulin deposition as well as increased circulating anti-oxidized LDL autoantibodies. Furthermore, we demonstrate that loss of leukocyte Bim interferes with lipid metabolism.

Methods

Bone marrow transplantation

All animal work was approved by regulatory authority of Leiden and performed in compliance with the Dutch government guidelines. LDLr$^{-/-}$ mice were obtained from the local animal breeding facility and Bim$^{-/-}$ mice from the Department of Biochemistry, Biosciences Research Institute from the University College Cork, Ireland. Male LDLr$^{-/-}$ mice (n=12) were housed in sterile individual ventilated cages with food and water ad libitum. The drinking water was supplied with antibiotics (83 mg/l ciprofloxacin and 67 mg/l Polymixin B) and 5 g/l sugar. The mice were exposed to a single dose of 9 Gy total body irradiation (0.19Gy/min, 200 kV, 4 mA) using an Andrex Smart 225 Röntgen source (YXLON International, Copenhagen, Denmark) one day before transplantation. Bone marrow was extracted from femurs and tibia of male Bim$^{-/-}$ and wild-type (WT) littermates. Irradiated LDLr$^{-/-}$ mice received either 5 x 10^6 Bim$^{-/-}$ bone marrow cells or 5 x 10^6 WT bone marrow cells via tail vein injection. After a recovery period of eight weeks the diet was changed from normal chow (RM3, Special Diet Services, Witham, Essex, UK) to Western type diet (WTD) containing 0.25% cholesterol and 15% cacao butter (Diet W, Special Diet Services, Witham, Essex, UK) for an additional ten weeks.

Cholesterol and triglyceride levels

Blood samples were taken by tail bleeding before bone marrow transplantation (BMT) and before and five weeks after start of Western type diet feeding and at the time of sacrifice. Hepatic lipids were extracted as described by Bligh and Dyer[26]. Total cholesterol, triglyceride and phospholipid content was measured spectrophotometrically using enzymatic procedures (Roche Diagnostics, Almere, The Netherlands) in serum and hepatic extracts.

Tissue harvesting and analysis

Sixteen weeks after BMT mice were anesthetized and perfused with PBS after which heart, aorta, spleen, thymus and mediastinal lymph nodes were isolated and stored in 4% formaldehyde solution or snap-frozen in liquid nitrogen and stored at -80ºC. Cryosections were prepared of aortic valves, spleen and liver and stained with hematoxylin and eosin (HE) and/or Oil Red 'O. The descending aorta was cut open longitudinally for *en face* analysis after staining with Oil Red 'O. Immunohistochemical stainings were performed for macrophage (MOMA-2, Sigma, Zwijndrecht, The Netherlands) and T cell (CD3, Immunologic, Duiven, The Netherlands) content in aortic root lesion, liver and/or spleen sections. Apoptotic cell content was quantified using terminal deoxytransferase dUTP nick-end labeling (TUNEL) kit (Roche Diagnostics) and presence of immunoglubulins using FITC

labeled rabbit anti-mouse Ig (DakoCytomation, Heverlee, Belgium). Lesion size and tissue morphology was analyzed using Leica image analysis system, consisting of a Leica DMRE microscope with camera and Leica Qwin Imaging software (Leica Ltd, Cambridge, UK). Fluorescent immunohistochemistry was analyzed on a Nikon Eclipse E600 using ImagePro 4.5 software.

Blood cell analysis and flow cytometry

Blood samples were taken by tail bleeding before BMT (week 0), before (week 6) and five weeks after start of Western type diet feeding (week 11) and at the time of sacrifice (week 16). Peritoneal leukocytes were isolated at the time of sacrifice by peritoneal lavage with 10 ml cold PBS. Whole blood and peritoneal lavage samples were analyzed on a Sysmex blood cell analyzer (XT-2000i, Sysmex Europe GmbH, Norderstedt, Germany). White blood cells (WBC) were isolated after erythrocyte lysis of whole blood samples obtained by orbital puncture. For flow cytometry, WBC and peritoneal leukocytes or single cell suspensions of spleen and lymph nodes were stained with fluorescently labelled antibodies from eBioscience (Halle-Zoersel, Belgium; CD3, CD4, CD8, CD11c, CD69, CD71, CD62L, CCR7 and MCH-II) and BD Pharmingen (Breda, The Netherlands; CD4, CD8 and CD45). Fluorescence-activated cell sorting (FACS) analysis was performed on a FACSCanto with FACSDiva software (BD Biosciences).

Macrophage apoptosis

Femurs and tibia were flushed with phosphate buffered saline (PBS) to isolate bone marrow. A single cell suspension was obtained by passing the bone marrow through a 70 µm nylon cell strainer (BD Falcon, Breda, The Netherlands). Bone marrow cells were differentiated into macrophages by culturing in 70% RPMI, supplemented with 20% FCS, glutamine, pyruvate, penicillin/streptomycin and non essential amino acids, and 30% M-CSF conditioned DMEM (obtained from L929 cells) for 7 days. Bone marrow derived macrophages (BMDM) were stimulated with 40 ug/ml Ox-LDL, cultured without growth factors (30% M-CSF conditioned DMEM) or in control medium for 24 hours. The macrophages were detached with accutase (PAA Laboratories GmbH, Cölbe, Germany), stained with Annexin V (ImmunoTools, Friesoythe, Germany) and propidium iodide (Sigma, Zwijndrecht, The Netherlands) and subsequently analyzed by flow cytometry using a FACSCalibur with CellQuest software (BD Biosciences).

Proliferation assay

Single cell suspensions from spleens of $Bim^{-/-}$ and WT BM transplanted mice were obtained by passing spleen tissue through a 70 µm nylon cell strainer (BD Falcon), after which splenocytes were washed with PBS. When indicated T cells were isolated from spleen and lymph nodes of $LDLr^{-/-}$ mice fed a Western type diet for 2 weeks using a Mouse T Lymphocyte Enrichment Set – DM ("purified T-cells"; BD Pharmingen, Breda, The Netherlands). Bone marrow cells of $Bim^{-/-}$ and

WT BM transplanted mice were differentiated into dendritic cells (DCs) by culturing in 70% IMDM, supplemented with 8% FCS, 2 mM glutamine, 100 U/ml penicillin/ streptomycin and 20 μM beta-mercaptoethanol, and 30% GM-CSF conditioned IMDM (obtained from R1 cells) for ten days. DCs were then stimulated with LPS (1 μg/ml), Ox-LDL (7.5 μg/ml) or left untreated for 24 h.
Splenocytes (2 x 10^5) or purified T-cells (6 x 10^5) were plated in triplicate in a round-bottom 96-well plate in RPMI containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol and incubated for 24 hours in the presence or absence of 2 μg/ml concanavalin A (splenocytes) or in the presence of stimulated or nonstimulated DCs (purified T-cells; cell ratio 1:10) at 37ºC. [^{3}H]-thymidine (0.5 μCi/well, GE Healthcare, Diegem, Belgium) was added and after incubation for another 24 hours samples were washed with PBS and cells were lysed in 0.1 M NaOH. [3H]-thymidine incorporation was measured by a liquid scintillation analyzer (Tri-Carb 2900R, Packard).

Detection of anti-Ox-LDL antibodies
An EIA/RIA high binding 96-well plate (Corning, Schiphol, The Netherlands) was coated with Ox-LDL (5 μg/ml) in a 50 mM Na_2CO_3/$NaHCO_3$ coating buffer (pH 9.6). IgM, IgG1 and IgG2a antibodies against Ox-LDL in serum were measured using an enzyme-linked immunosorbent assay (ELISA) Ig detection kit (Zymed Laboratories, San Francisco, CA, USA) according to the manufacturer's protocol.

Statistical analysis
Values are expressed as mean ± SEM or presented as mean + upper limit of the SEM. Analysis to compare two groups was performed by two-tailed Student's t-test. P-values less than 0.05 are considered significant.

Results
Serum lipid levels are reduced in Bim$^{-/-}$ BM transplanted mice
The role of leukocyte Bim in atherogenesis was studied in chimeras generated by transplanting bone marrow (BM) from Bim$^{-/-}$ and WT littermates to irradiated LDLr$^{-/-}$ recipients. First we analyzed whether leukocyte Bim deletion in western type diet fed LDLr$^{-/-}$ mice influenced serum total cholesterol (TC) and triglyceride (TG) levels. To our surprise TC and TG levels of Bim$^{-/-}$ BM transplanted mice were reduced by a very significant 30-48% compared to controls both after 5 and after 10 weeks of western type diet feeding ($p<0.05$, fig. 1A and B). The reduction in TC was clearly induced by Western type diet since there was no difference between groups when mice were fed a chow diet, eight weeks after BMT. However, at this time point serum TG levels had already dropped by 35% ($p<0.05$). Body weight was comparable between the two groups of mice (39.4 ± 1.7 g vs. 38.9 ± 1.8 g for WT and Bim$^{-/-}$ BM transplanted mice respectively), suggesting that the decreased TC and TG levels were not caused by differences in the general metabolic state.
Reduction of serum lipid levels neither did translate in nor was caused by perturbed

hepatic lipid handling as we did not observe differences in hepatic lipid content (fig. 1C and D). Livers of Bim$^{-/-}$ BM transplanted mice did however show an increased leukocyte content, consisting for a substantial part of T cells, suggestive of increased hepatic inflammation (fig 1E and F).

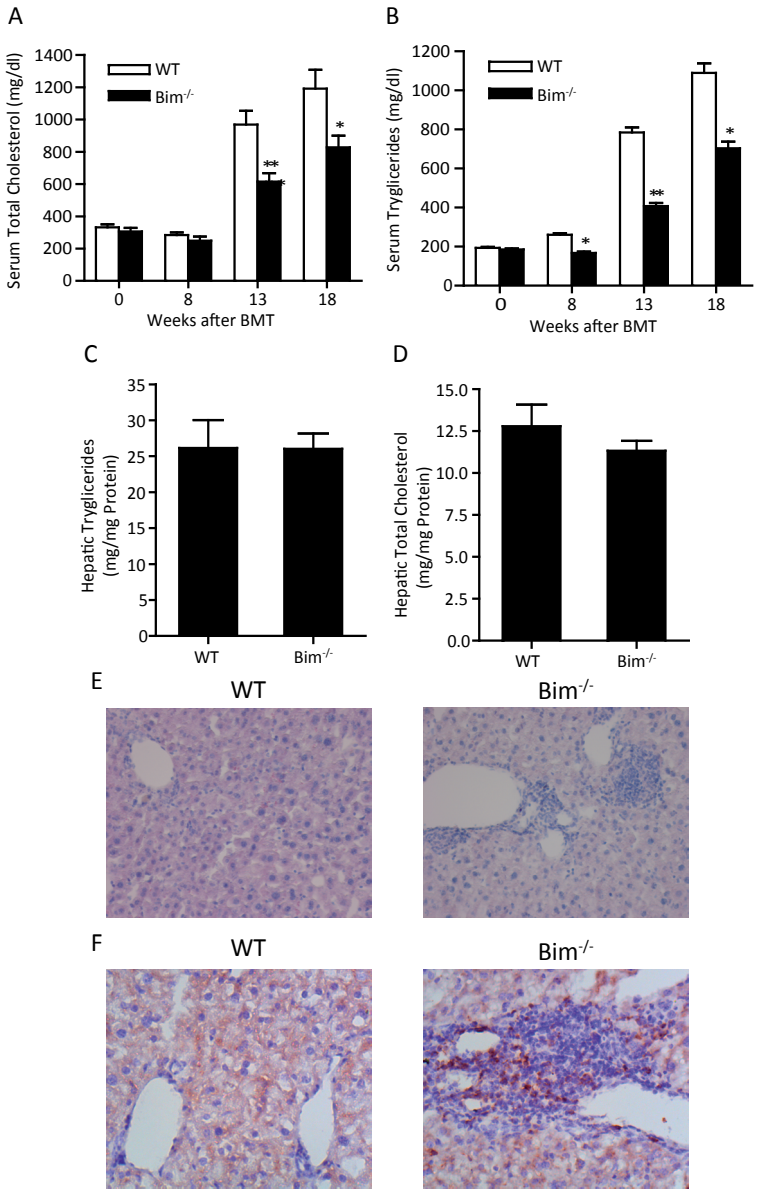

Figure 1. Serum lipid levels are reduced. Circulating total cholesterol (TC) levels are reduced after Western type diet feeding of Bim$^{-/-}$ BM transplanted LDLr$^{-/-}$ mice (A), circulating triglyceride (TG) levels are reduced independent of diet (B). The reductions in lipid levels are not caused by changes in hepatic TC and TG content (C and D). Livers of Bim$^{-/-}$ chimeras show increased cell influx, consisting partly of T cells. Representative picture of a HE (E) and CD3 stained (brown) cryosections (F). (*p<0.05, **p<0.01)

Loss of leukocyte Bim in LDLr$^{-/-}$ mice affects T cell levels and characteristics

As Bim is instrumental in lymphocyte development and T cell response, we assessed these characteristics in Bim$^{-/-}$ BM transplanted LDLr$^{-/-}$ mice. The spleen of Bim$^{-/-}$ BM recipients was enlarged (+81% in relative weight; p<0.001, fig. 2A) compared to WT BM recipients. Thymus weight and mediastinal lymph node (MLN) total cell number were unaltered (fig 2B and C). Total white blood cells in Bim$^{-/-}$ BM transplanted mice were increased by almost two-fold, which was mainly attributable to a 90% increase in circulating lymphocyte numbers (p<0.01, fig. 3A). Next, T cell content and activation in spleen and MLN was analyzed. Compared to controls MLN from Bim$^{-/-}$ BM recipients were slightly enriched in CD4^{+} and CD8^{+} T cells (p<0.01, fig. 3B). Relative spleen T cell content was unchanged (fig. 3C), indicating that the different leukocyte subsets were enhanced proportionally. However both CD4^{+} and CD8^{+} T cell subsets were found to be more activated in Bim$^{-/-}$ BM transplanted mice in comparison with WT transplanted mice as judged from the enhanced expression of activation markers CD69 and CD71 (p<0.05, fig. 3D and E).

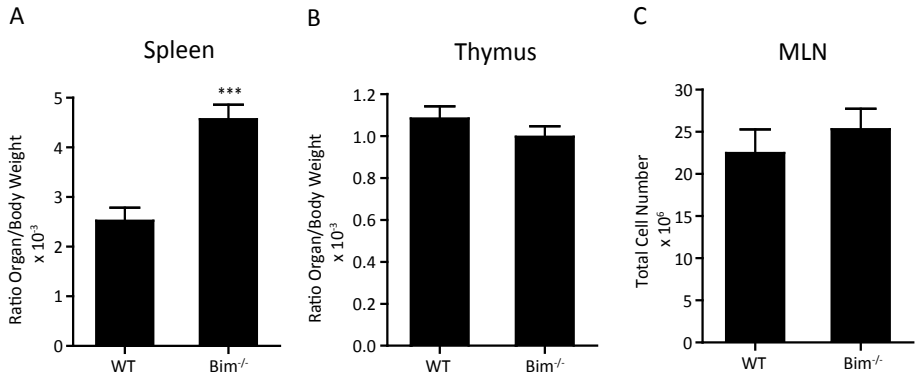

Figure 2. Organ weights in Bim$^{-/-}$ versus Bim$^{+/+}$ bone marrow transplanted LDLr$^{-/-}$ mice. Spleen weight was increased in Bim$^{-/-}$ BM recipients (A), while thymus weight was unaltered (B). Values are expressed as organ weight relative to total body weight. (***p<0.001)

In keeping with this finding, basal proliferation of unstimulated splenocytes derived from Bim$^{-/-}$ BM recipients, as measured by [^{3}H]-thymidine incorporation, was increased by 38% (p<0.05, fig. 4A). Concanavalin A induced proliferation was however not affected in Bim$^{-/-}$ splenocytes (fig. 4B), indicating that Bim deficiency does not alter the splenocyte proliferation capacity. Next, we examined the ability of WT and Bim$^{-/-}$ dendritic cells to stimulate T cell proliferation. Bone marrow cells from Bim$^{-/-}$ and WT transplanted mice were differentiated into dendritic cells (DCs) by growth factor stimulation. DCs were subsequently matured in the presence of lipopolysaccharide (LPS) or Ox-LDL to stimulate antigen presentation. Proliferation of T cells isolated from western type diet fed LDLr$^{-/-}$ mice was measured after co-incubation for 24h with matured DCs. Proliferation of T cells incubated with immature WT or Bim$^{-/-}$ DCs was essentially similar as was proliferation of T cells incubated with Ox-LDL pulsed DCs (fig. 4C). However Bim$^{-/-}$ DCs stimulated with LPS were able to stimulate T cell proliferation to a slightly higher extent than unstimulated Bim$^{-/-}$ DCs

while for WT DCs no significant increase in T cell proliferation was observed after LPS stimulation ($p<0.05$, fig. 4C).

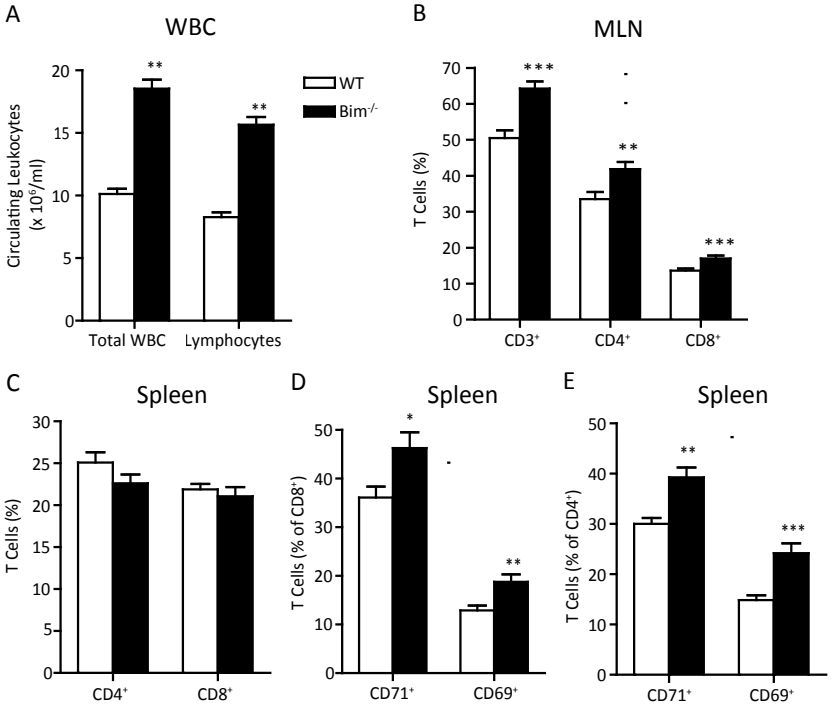

Figure 3. Effect of hematopoietic Bim$^{-/-}$ deficiency on lymphoid organ T cell content and activation. Total white blood cell numbers were largely increased in Bim$^{-/-}$ chimeras, mainly due to the large increase in lymphocyte counts (A). Both CD4$^+$ and CD8$^+$ T cell levels were elevated in mediastinal lymph nodes (MLN; B), but not in spleen (C). However in spleen a larger portion of CD4$^+$ and CD8$^+$ T cells were activated as shown by increased CD69 and CD71 expression (D and E). (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

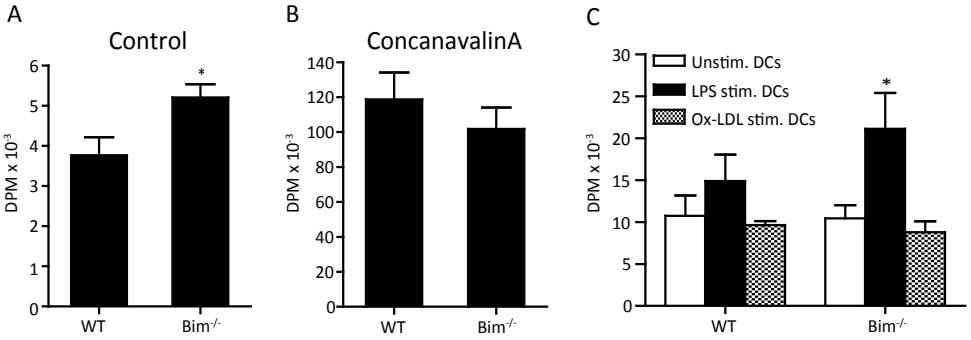

Figure 4. Proliferative capacity of Bim deficient splenocytes and T cells. Basal proliferation of splenocytes is enhanced after leukocyte Bim deletion (A), while that of concanavalin A stimulated splenocytes is unchanged (B). Proliferation of T cells isolated from LDLr$^{-/-}$ mice fed a Western type diet for two weeks was increased after incubation with Bim$^{-/-}$ dendritic cells (DCs) stimulated with LPS, but not after incubation with nonstimulated Bim$^{-/-}$ DCs or DCs stimulated with Ox-LDL as compared to T cells incubated with stimulated or nonstimulated WT DCs (C). (*$p<0.05$)

Leukocyte Bim deficiency affects circulating myeloid cell and lymph node DCs

In addition to elevated circulating lymphocyte numbers, monocyte/macrophage and neutrophil levels in blood were elevated by 87% and 42%, respectively ($p<0.05$, fig 5A). Mediastinal lymph nodes surprisingly showed a reduced total dendritic cell (DC) content (10.0 ± 0.8 % in $Bim^{-/-}$ BM transplanted mice vs. 7.6 ± 0.7% in WT BM transplanted mice; $p<0.05$, fig. 5B). $MHCII^{+}$ DCs were reduced to the same extent as total DCs, suggesting that Bim deficiency does not influence activation of DCs in $LDLr^{-/-}$ mice. Relative DC content in spleen remained unchanged (fig. 5C).

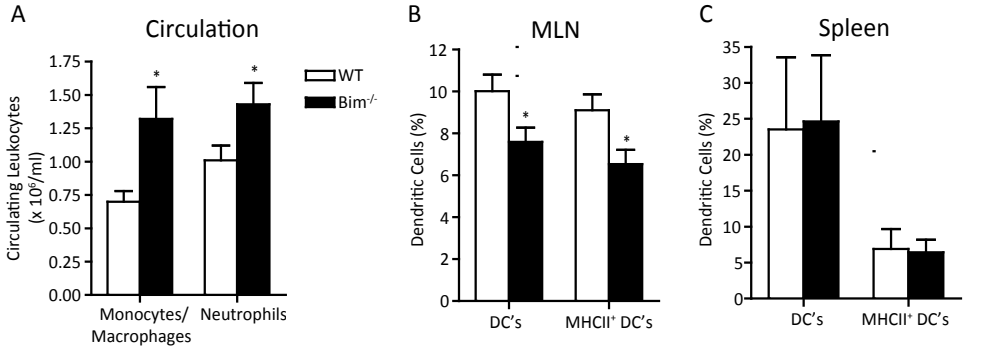

Figure 5. Consequences of Bim deletion on circulating, splenic and MLN myeloid cell content. Circulating monocytes/macrophages and neutrophils are increased in Bim deficient $LDLr^{-/-}$ mice fed a Western type diet for ten weeks (A). Total DC and $MHCII^{+}$ DC content is reduced after Bim deletion in mediastinal lymph nodes (MLN; B) but not in spleen (C). (*$p<0.05$)

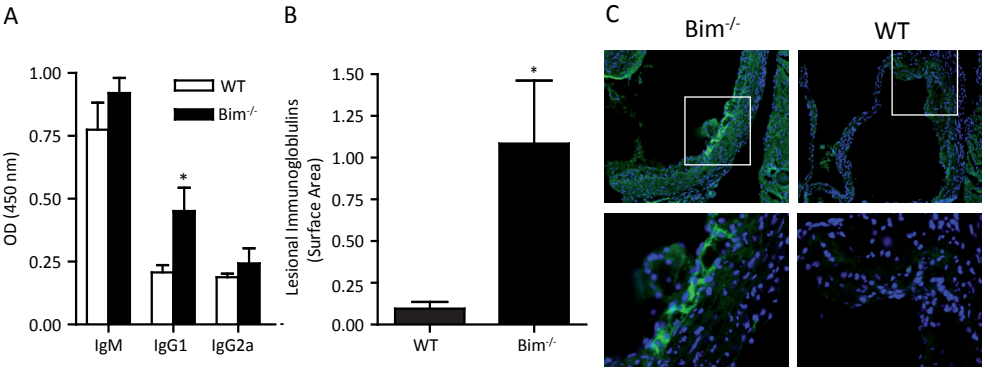

Figure 6. Effect of leukocyte Bim deficiency on humoral immune response. Ox-LDL specific antibody titers in serum after 10 weeks of Western type diet feeding were measured by ELISA. IgG1 antibody levels were increased while IgM and IgG2a antibody levels were unchanged suggestive of a Th2 shifted T-cell response (A). Total immunoglobulin (Ig) deposition in aortic root lesions was massively increased in $Bim^{-/-}$ transplanted mice (B). Representative cryosections showing overt staining for total Ig (green); nuclei are stained with DAPI (blue) (C). (*$p<0.05$)

Leukocyte Bim deficiency affects the humoral immune response in $LDLr^{-/-}$ mice

Apart from affecting T cell homeostasis, loss of Bim was seen to be associated with impaired deletion of autoreactive B cells. The resulting accumulation of these cells and derived autoreactive antibodies *in vivo*[15] can promote autoimmunity[12]. We therefore measured Ox-LDL directed autoantibody titers in serum. IgG1 anti-Ox-

LDL antibodies were increased by more than two-fold ($p<0.05$, fig. 6A) in Bim$^{-/-}$ BM transplanted mice compared to controls, but no differences in IgM and IgG2a anti-Ox-LDL auto-antibodies were detected. Consequently the IgG1/IgG2a ratio, a measure of Th2/Th1 polarized T-cell response, was sharply increased in Bim$^{-/-}$ chimeras from 1.19 ± 0.22 for WT BM recipients to 2.84 ± 0.71 for Bim$^{-/-}$ BM recipients ($p=0.06$). Importantly, staining for deposits of total immunoglobulins (Ig) in lesions revealed the striking presence of Ig complexes in lesions of Bim$^{-/-}$ chimeras but not littermate controls (0.10 ± 0.04 % versus 1.08 ± 0.04%; $p<0.05$, fig. 6B and C).

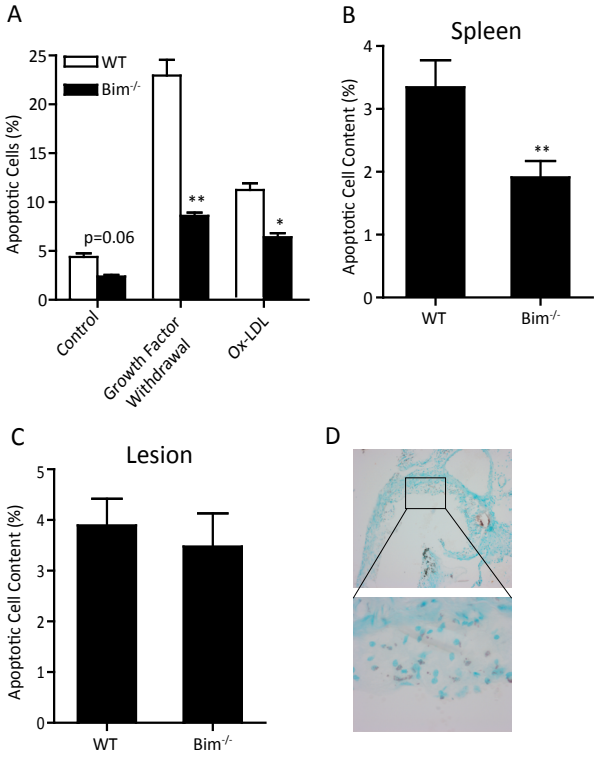

Figure 7. Apoptotic cell death in leukocyte Bim deficient LDLr$^{-/-}$ mice. Apoptosis of bone marrow derived macrophages is reduced in Bim$^{-/-}$ transplanted mice at basal level, after growth factor withdrawal, and after stimulation with Ox-LDL (40 µg/ml) (A). Spleen apoptotic cell content is decreased by 45% in Bim$^{-/-}$ chimeras (B), but is unaffected in atherosclerotic lesions (C). Representative pictures of TUNEL stained aortic root cryosection (D). (*$p<0.05$, **$p<0.01$)

Apoptotic cell death is affected by loss of leukocyte Bim

Loss of Bim in bone marrow derived macrophages (BMDM) resulted in decreased sensitivity to apoptotic cell death in response to a number of stimuli. Basal apoptosis levels were already reduced in Bim$^{-/-}$ compared to WT BMDM (45%, $p=0.06$, fig. 7A). While in WT BMDM apoptosis increased from 4.39 ± 0.81% to 22.94 ± 3.61% upon growth factor withdrawal, in Bim$^{-/-}$ BMDM apoptosis increased from 2.39 ± 0.37 to only 8.59 ± 0.70% ($p<0.01$, fig. 7A). OxLDL induced apoptotic cell death was 43% less in Bim$^{-/-}$ compared to WT BMDM ($p<0.05$), but interestingly the relative increase over non-stimulated cells was similar in WT and Bim$^{-/-}$ BMDM (fig. 7A). Since we observed profound splenomegaly in Bim$^{-/-}$ BM recipients we measured apoptotic cell content in spleens, which appeared to be significantly lower in Bim$^{-/-}$ BM compared to WT BM transplanted mice (3.34 ± 0.43 vs. 1.84 ± 0.29 % of total splenocytes for WT and Bim$^{-/-}$ BM transplanted mice respectively, $p<0.01$, fig. 7B).

In striking contrast, apoptotic cell content of atherosclerotic lesions remained unchanged (fig. 7C and D).

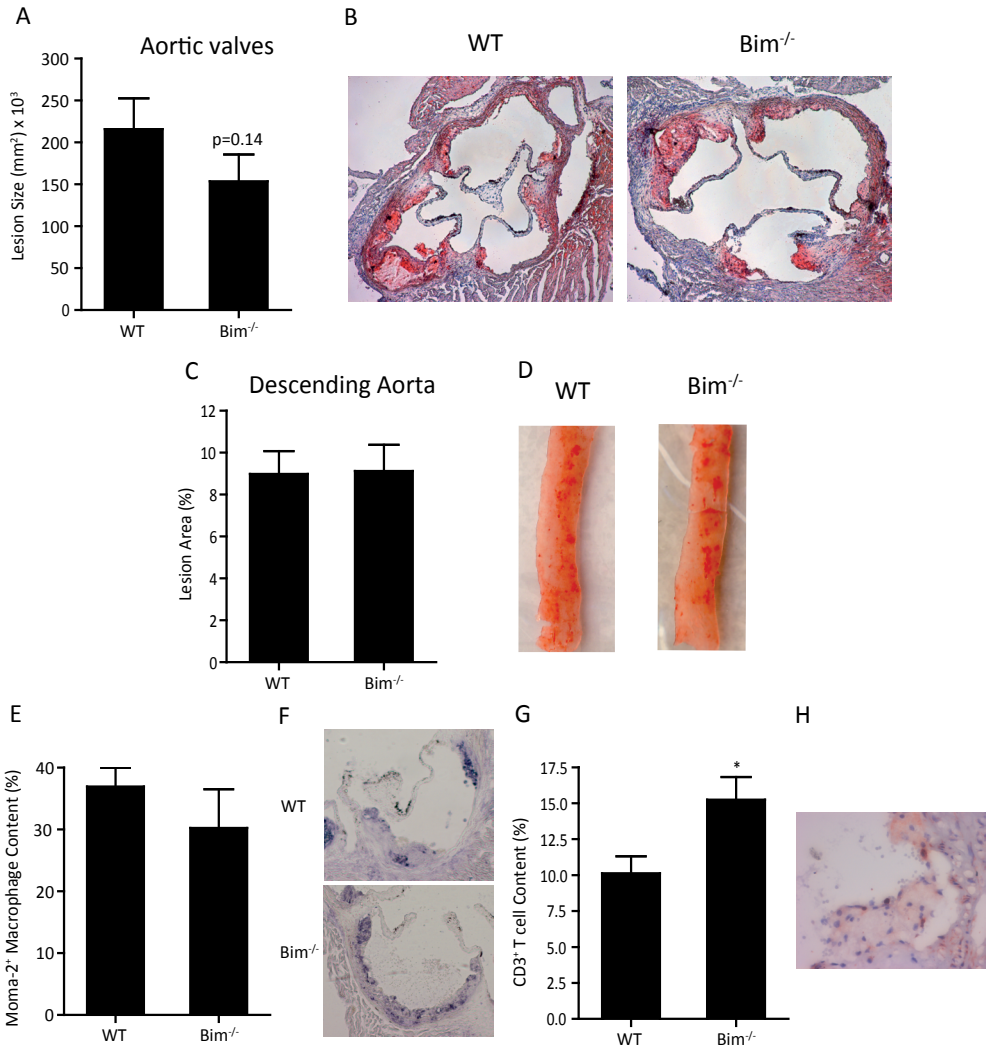

Figure 8. Atherosclerotic lesion size and composition. Lesion size in aortic root and descending aorta were unchanged in LDLr$^{-/-}$ mice with hematopoietic Bim deficiency (A and C respectively, with representative pictures [B and D]) as was Moma-2^{+} macrophage content (E, with representative pictures [F]). CD3^{+} T cell content was increased in Bim$^{-/-}$ transplanted mice (G). Representative picture of a CD3 stained (brown) cryosection (H). (*p<0.05)

Atherosclerotic lesion size and stability are not changed after loss of leukocyte Bim

After ten weeks of Western type diet feeding mice were sacrificed and the aortic root and descending aorta were isolated and analyzed for size and composition. In keeping with elevated T cell levels in circulation and lymphoid organs T cell content in the atherosclerotic lesions (intima and adventitia) was increased by 51% from 10.1 ± 1.2 per section in WT BM recipients to 15.3 ± 1.6 per section in Bim$^{-/-}$ BM

transplanted recipients ($p<0.05$, fig. 8G and H). We did not observe any significant differences in lesion size in aortic root between groups (fig. 8A and B). In agreement *en face* analysis of the descending aorta showed no differences in atherosclerotic lesion area as well (fig. 8C and D). Regarding composition, lesional moma-2 positive macrophage content in $Bim^{-/-}$ did not differ from that in WT BM transplanted mice (fig. 8E and F). Collagen and necrotic core were virtually absent in the early lesions of WT and $Bim^{-/-}$ BM transplanted mice.

Discussion

Bim was demonstrated to be essential for apoptosis of various leukocyte subsets, including T and B cells, dendritic cells, macrophages and granulocytes[12,13,27]. As these cell types are all shown to be present in atherosclerotic lesions and to contribute to disease development, a role for Bim in lesional leukocyte apoptosis and atherogenesis may be anticipated. Therefore we generated $LDLr^{-/-}$ mice with Bim deficiency in hematopoietic cells by bone marrow transplantation and addressed effects of Bim deficiency on lesion development. The most profound consequences of Bim deficiency following BMT were observed on T cell homeostasis. Lymphocytes, although in atherosclerotic lesions less abundantly present than macrophages, are importantly involved in regulating immune responses in lesions[20,28]. Lymphocyte deficiency in mice has previously been demonstrated to result in reduced atherosclerotic lesion size[17,18] and transfer of $CD4^+$ T cells in immunodeficient mice was seen to aggravate atherosclerosis[19]. In keeping with previous studies[12] we show markedly elevated circulating lymphocyte levels in $Bim^{-/-}$ BM transplanted $LDLr^{-/-}$mice. Furthermore, mediastinal lymph node $CD4^+$ and $CD8^+$ T cell content is slightly increased and $LDLr^{-/-}$ mice suffer from considerable splenomegaly after leukocyte Bim deletion. Our results are consistent with previous observations that systemic Bim deficient mice have enlarged spleens and highly elevated lymphocyte content in circulation and lymphoid organs[12], although as expected in systemic Bim deficiency the above-mentioned differences were more pronounced. As suggested by Bouillet and Hildeman and colleagues this overt lymphocytosis may be caused by impaired apoptosis of autoreactive thymocytes and activated T cells for which Bim has been demonstrated to be essential[14,16]. In agreement with the latter, both $CD4^+$ and $CD8^+$ splenic T cells are more activated in $Bim^{-/-}$ BM recipients than in control mice, both at an absolute (% of total splenocytes) and at a relative level (% of the respective T cell subset). The observed elevated T cell numbers are reflected in increased T cell content in atherosclerotic lesions indicating an enhanced infiltration of T cells from circulation into the lesions. Decreased apoptotic cell death in spleen was accompanied by a slightly increased basal splenocyte proliferation in $Bim^{-/-}$ BM transplanted mice. The proliferation capacity of $Bim^{-/-}$ splenocytes was not affected. Although increased proliferation of $Bim^{-/-}$ splenocytes has not been described before, Bim interacts with anti-apoptotic Bcl-2 family members, which in turn were seen to influence cell cycle entry[29,30]. Interestingly, T-cell proliferation was increased after stimulation with LPS but not Ox-LDL pulsed $Bim^{-/-}$ DCs compared

to immature DCs. This effect was absent in WT DCs. Previously loss of Bim in DCs was seen to decrease cell death and to promote proliferation of antigen-specific T cells[27]. We extend these findings by showing that pulsed Bim$^{-/-}$ DCs are able to enhance proliferation of nonspecific T cells as well.

In addition to T cell homeostasis and activation Bim was previously reported to be necessary for apoptosis of autoreactive B cells[15] and to prevent autoimmunity[12]. Atherosclerosis is currently recognized as a lipid driven inflammatory process with features of autoimmune disease[20-22], implicating T cell responses to auto-antigens, such as Ox-LDL and heat shock proteins (HSP)[22]. In fact, Ox-LDL specific antibodies have been detected in atherosclerosis-prone ApoE$^{-/-}$ mice[31], in human and rabbit serum and in atherosclerotic lesions[32]. Moreover, a subset of T cells present in human lesions was demonstrated to be Ox-LDL specific[33]. In the present study we observed markedly elevated Ox-LDL antibody levels in serum of Bim$^{-/-}$ BM transplanted mice. In addition, total immunoglobulin deposition in atherosclerotic lesions of Bim$^{-/-}$ BM recipients was dramatically increased. Generally, these auto-antibodies directed against Ox-LDL are thought to be atheroprotective as several studies have demonstrated decreased lesion formation after immunization with modified LDL[34-37]. Furthermore IgG antibodies against modified LDL and oxidized phospholipids were increased in immunized LDLr$^{-/-}$ mice and correlated with decreased lesion formation[37]. Furthermore B cell associated immunity was shown to be protective in atherosclerosis as splenectomy increased lesion size of ApoE$^{-/-}$ mice which could be counteracted by transfer of spleen derived B cells[38]. The observed increase in anti-Ox-LDL antibodies in serum of Bim$^{-/-}$ BM recipients is mainly due to elevated anti-Ox-LDL IgG1 levels, whereas anti-Ox-LDL IgM and IgG2a isotypes were not affected. This may point to a shift towards Th2 type immune responses, which may by itself influence the progression of atherosclerosis[20]. Both mouse and human studies have demonstrated a predominant Th1 response in atherosclerotic plaques, with Th1 cytokines such as IFNγ, IL2 and TNFα abundantly present within the lesion[39,40]. Th2 cells, in contrast, produce IL4, IL5, IL10 and IL13 which promote antibody formation and inhibit pro-inflammatory cytokine expression and are thought to protect against atherosclerosis[20,21].

In agreement with previous studies[12], not only circulating lymphocytes numbers but also that of monocyte/macrophages and neutrophils were increased in Bim$^{-/-}$ BM transplanted mice. Surprisingly, this does not translate into increased plaque macrophage content or elevated relative macrophage and DC content in lymphoid organs, except for a slight relative decrease in mediastinal lymph node DCs. Since loss of Bim has been demonstrated to increase half life and proliferation of DCs[27] our result may indicate that either trafficking of antigen presenting DCs into lymph nodes is impaired or that uptake of antigen by DCs and subsequent DC maturation is reduced in Bim$^{-/-}$ BM recipients compared to WT BM recipients.

Absence of Bim decreased spontaneous apoptosis of bone marrow derived macrophages as well as apoptosis induced by growth factor withdrawal. These results are consistent with previous studies showing decreased apoptosis in Bim deficient

T cells[12], DCs[27] and granulocytes[13] in response to various stimuli. In addition we identify Bim as a regulator of Ox-LDL induced apoptosis of macrophages. However while spleen apoptotic cell content was decreased in Bim$^{-/-}$ BM recipients as expected, we did not observe any differences in atherosclerotic lesion apoptotic cell content, predominantly representing macrophages and foam cells. Bim dependent pro-apoptotic effects on T cells, which are present at much lower numbers in atherosclerotic lesions[20], are probably not detectable.

Despite marked effects on T cell levels and characteristics, humoral immune response and myeloid subset levels, leukocyte Bim deficiency in LDLr$^{-/-}$ mice did not alter atherosclerotic lesion size or stability as assessed at two different vascular sites, early atherogenesis in the descending aorta and intermediate stage atherosclerosis in the aortic root valves. Apart from an increased lesional T cell content no differences were observed in aortic root lesion composition. Surprisingly leukocyte Bim deficiency following BMT led to a striking reduction in serum lipids, e.g. total cholesterol and triglycerides, in LDLr$^{-/-}$ mice. For triglycerides this effects was independent of the diet. Decreased serum lipid levels were not caused by differences in hepatic lipid content. To what extent hepatic inflammation represented by T-cells which were elevated in livers of Bim$^{-/-}$ BM transplanted mice had contributed to the hypolipidemia remains to be determined. Nevertheless, such involvement of Bim in hepatic lipid metabolism has not yet been described thus far and warrants further study..

In conclusion, we show here that leukocyte Bim deficiency in LDLr$^{-/-}$ mice results in increased activated T-cell content in circulation, lymphoid organs and atherosclerotic lesions, in increased levels of autoreactive antibodies directed against oxidized LDL in circulation and in substantial immunoglobulin deposition in atherosclerotic lesions. Collectively these pro- and anti-atherogenic effects of leukocyte Bim deficiency, summarized in table 1, together with the surprising reduction in serum lipid levels likely counterbalance so that atherosclerosis development remains unaffected.

Table 1. Pro- and anti-atherogenic consequences of leukocyte Bim deficiency in LDLr$^{-/-}$ mice.

Pro-atherogenic		**Anti-atherogenic**	
Circulating and lymph node T cell levels	↑	Serum cholesterol	↓
T cell activation	↑	Serum triglycerides	↓
Circulating Monocyte/macrophage and neutrophil levels	↑	Ox-LDL antibodies	↑
Apoptosis (depending on cell type)	↓	Apoptosis (depending on cell type)	↓

References

1. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999; 340(2):115-26.
2. Lutgens E, de Muinck ED, Kitslaar PJ, Tordoir JH, Wellens HJ, Daemen MJ. Biphasic pattern of cell turnover characterizes the progression from fatty streaks to ruptured human atherosclerotic plaques. Cardiovasc Res. 1999; 41(2):473-9.
3. van Vlijmen BJ, Gerritsen G, Franken AL, Boesten LS, Kockx MM, Gijbels MJ, Vierboom MP, van Eck M, van De Water B, van Berkel TJ, Havekes LM. Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. Circ Res. 2001; 88(8):780-6.
4. Stoneman V, Braganza D, Figg N, Mercer J, Lang R, Goddard M, Bennett M. Monocyte/macrophage suppression in CD11b diphtheria toxin receptor transgenic mice differentially affects atherogenesis and established plaques. Circ Res. 2007; 100(6):884-93.
5. Schrijvers DM, De Meyer GR, Kockx MM, Herman AG, Martinet W. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. Arterioscler Thromb Vasc Biol. 2005; 25(6):1256-61.
6. Marsden VS, Strasser A. Control of apoptosis in the immune system: Bcl-2, BH3-only proteins and more. Annu Rev Immunol. 2003; 21:71-105.
7. Liu J, Thewke DP, Su YR, Linton MF, Fazio S, Sinensky MS. Reduced macrophage apoptosis is associated with accelerated atherosclerosis in low-density lipoprotein receptor-null mice. Arterioscler Thromb Vasc Biol. 2005 Jan;25(1):174-9.
8. Thorp E, Li Y, Bao L, Yao PM, Kuriakose G, Rong J, Fisher EA, Tabas I. Brief report: increased apoptosis in advanced atherosclerotic lesions of Apoe-/- mice lacking macrophage Bcl-2. Arterioscler Thromb Vasc Biol. 2009; 29(2):169-72.
9. O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, Huang DC. Bim: a novel member of the Bcl-2 family that promotes apoptosis. EMBO J. 1998; 17(2):384-95.
10. Hsu SY, Lin P, Hsueh AJ. BOD (Bcl-2-related ovarian death gene) is an ovarian BH3 domain-containing proapoptotic Bcl-2 protein capable of dimerization with diverse antiapoptotic Bcl-2 members. Mol Endocrinol. 1998; 12(9):1432-40.
11. Strasser A. The role of BH3-only proteins in the immune system. Nat Rev Immunol. 2005; 5(3):189-200.
12. Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Köntgen F, Adams JM, Strasser A. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science. 1999; 286(5445):1735-8.
13. Villunger A, Scott C, Bouillet P, Strasser A. Essential role for the BH3-only protein Bim but redundant roles for Bax, Bcl-2, and Bcl-w in the control of granulocyte survival. Blood. 2003; 101(6):2393-400.
14. Bouillet P, Purton JF, Godfrey DI, Zhang LC, Coultas L, Puthalakath H, Pellegrini M, Cory S, Adams JM, Strasser A. BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. Nature. 2002; 415(6874):922-6.
15. Enders A, Bouillet P, Puthalakath H, Xu Y, Tarlinton DM, Strasser A. Loss of the pro-apoptotic BH3-only Bcl-2 family member Bim inhibits BCR stimulation-induced apoptosis and deletion of autoreactive B cells. J Exp Med. 2003; 198(7):1119-26.
16. Hildeman DA, Zhu Y, Mitchell TC, Bouillet P, Strasser A, Kappler J, Marrack P. Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. Immunity. 2002; 16(6):759-67.
17. Reardon CA, Blachowicz L, White T, Cabana V, Wang Y, Lukens J, Bluestone J, Getz GS. Effect of immune deficiency on lipoproteins and atherosclerosis in male apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol. 2001; 21(6):1011-6.
18. Dansky HM, Charlton SA, Harper MM, Smith JD. T and B lymphocytes play a minor role in atherosclerotic plaque formation in the apolipoprotein E-deficient mouse. Proc Natl Acad Sci U S A. 1997; 94(9):4642-6.
19. Zhou X, Nicoletti A, Elhage R, Hansson GK. Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice. Circulation. 2000; 102(24):2919-22.
20. Hansson GK, Libby P, Schönbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. Circ Res. 2002; 91(4):281-91.
21. Binder CJ, Chang MK, Shaw PX, Miller YI, Hartvigsen K, Dewan A, Witztum JL. Innate and acquired

immunity in atherogenesis. Nat Med. 2002; 8(11):1218-26.
22. Nilsson J, Hansson GK. Autoimmunity in atherosclerosis: a protective response losing control? J Intern Med. 2008; 263(5):464-78.
23. Mallat Z, Ait-Oufella H, Tedgui A. Regulatory T-cell immunity in atherosclerosis. Trends Cardiovasc Med. 2007; 17(4):113-8
24. Ait-Oufella H, Salomon BL, Potteaux S, Robertson AK, Gourdy P, Zoll J, Merval R, Esposito B, Cohen JL, Fisson S, Flavell RA, Hansson GK, Klatzmann D, Tedgui A, Mallat Z. Natural regulatory T cells control the development of atherosclerosis in mice. Nat Med. 2006; 12(2):178-80.
25. Gotsman I, Grabie N, Gupta R, Dacosta R, MacConmara M, Lederer J, Sukhova G, Witztum JL, Sharpe AH, Lichtman AH. Impaired regulatory T-cell response and enhanced atherosclerosis in the absence of inducible costimulatory molecule. Circulation. 2006; 114(19):2047-55.
26. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 1959; 37(8):911-7.
27. Chen M, Huang L, Wang J. Deficiency of Bim in dendritic cells contributes to overactivation of lymphocytes and autoimmunity. Blood. 2007; 109(10):4360-7.
28. Hansson GK, Robertson AK, Söderberg-Nauclér C. Inflammation and atherosclerosis. Annu Rev Pathol. 2006; 1:297-329.
29. O'Reilly LA, Huang DC, Strasser A. The cell death inhibitor Bcl-2 and its homologues influence control of cell cycle entry. EMBO J. 1996; 15(24):6979-90.
30. Mazel S, Burtrum D, Petrie HT. Regulation of cell division cycle progression by bcl-2 expression: a potential mechanism for inhibition of programmed cell death. J Exp Med. 1996; 183(5):2219-26.
31. Palinski W, Hörkkö S, Miller E, Steinbrecher UP, Powell HC, Curtiss LK, Witztum JL. Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. J Clin Invest. 1996; 98(3):800-14.
32. Ylä-Herttuala S, Palinski W, Butler SW, Picard S, Steinberg D, Witztum JL. Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL. Arterioscler Thromb. 1994; 14(1):32-40.
33. Stemme S, Faber B, Holm J, Wiklund O, Witztum JL, Hansson GK. T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. Proc Natl Acad Sci U S A. 1995; 92(9):3893-7.
34. Ameli S, Hultgårdh-Nilsson A, Regnström J, Calara F, Yano J, Cercek B, Shah PK, Nilsson J. Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits. Arterioscler Thromb Vasc Biol. 1996; 16(8):1074-9.
35. George J, Afek A, Gilburd B, Levkovitz H, Shaish A, Goldberg I, Kopolovic Y, Wick G, Shoenfeld Y, Harats D. Hyperimmunization of apo-E-deficient mice with homologous malondialdehyde low-density lipoprotein suppresses early atherogenesis. Atherosclerosis. 1998; 138(1):147-52.
36. Freigang S, Hörkkö S, Miller E, Witztum JL, Palinski W. Immunization of LDL receptor-deficient mice with homologous malondialdehyde-modified and native LDL reduces progression of atherosclerosis by mechanisms other than induction of high titers of antibodies to oxidative neoepitopes. Arterioscler Thromb Vasc Biol. 1998; 18(12):1972-82.
37. Zhou X, Caligiuri G, Hamsten A, Lefvert AK, Hansson GK. LDL immunization induces T-cell-dependent antibody formation and protection against atherosclerosis. Arterioscler Thromb Vasc Biol. 2001; 21(1):108-14.
38. Caligiuri G, Nicoletti A, Poirier B, Hansson GK. Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. J Clin Invest. 2002; 109(6):745-53.
39. Zhou X, Paulsson G, Stemme S, Hansson GK. Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. J Clin Invest. 1998; 101(8):1717-25.
40. Frostegård J, Ulfgren AK, Nyberg P, Hedin U, Swedenborg J, Andersson U, Hansson GK. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. Atherosclerosis. 1999; 145(1):33-43.