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ABSTRACT

Atherogenesis is equally influenced by dyslipidemia and inflammation, and both innate and adaptive immune responses have been shown to modulate atherosclerotic lesion formation. In fact immune mechanisms may even be decisive in the clinical outcome. The immune responses involved are prominently directed against oxidation-specific epitopes on oxidized low-density lipoproteins (OxLDL) as well as on apoptotic cells, which are both present in atherosclerotic lesions. Oxidation-specific epitopes are generated as a consequence of lipid peroxidation, and represent danger signals that are recognized by IgM natural antibodies (NAbs), which are produced by innate B1 cells, in humans and mice. The exact role of B cell populations in atherogenesis is still unclear, though it has been shown that B cell depletion by CD20 antibody treatment, which severely depletes B2 cells and dramatically reduces IgG antibodies but affects B1 cells and IgM antibodies to a lesser extent, significantly reduces lesion formation. Mice deficient in serum IgM antibodies, which lack NAbs, have significantly accelerated lesion formation, and we hypothesize that B1 cells and the natural antibodies they secrete protect from atherosclerosis, whereas the adaptive humoral immunity mediated through B2 cells and IgG antibodies act pro-atherogenic. Indeed, in a recent publication Kyaw et al. showed that the B1a lymphocyte subpopulation is atheroprotective, as the phenotype of aggravated lesion formation in splenectomized ApoE deficient mice was reversed by adoptive transfer of B1a cells. The possibility to study the role of the entire B1 cell pool could be achieved by specific depletion of B2 cells with neutralizing antibodies that are directed against the BAFF-BAFFR interaction. BAFF-BAFFR interaction is crucial for the survival of B2 cells, while BAFF inhibition in vivo leaves natural antibody producing B1 cells unaffected. In this thesis I primarily focused on the function of the BAFFR on B1 cells and could indicate a role of the BAFF-BAFFR interaction with respect to spontaneous IgM secretion. I could show that neutralization of BAFF signaling leads to increased production of IgM antibodies by B1 cells. In addition, I could show that macrophages stimulate IgM

secretion of B1 cells and that this stimulation is dependent on cell contact. In this regard, I observed the hitherto unknown fact that macrophages express the BAFFR.

ZUSAMMENFASSUNG

Atherosklerose wird sowohl von Fettstoffwechselstörungen als auch durch Entzündung beeinflusst. Dabei wird die Bildung von Läsionen durch angeborene und adaptive Immunmechnanismen moduliert. Diese Immunantwort richtet sich in erster Linie gegen oxidationsspezifische Epitope auf oxidierten Lipoproteinen niedriger Dichte (LDL) und apoptotischen Zellen, die in atherosklerotischen Läsionen akkumulieren. Oxidationsspezifische Epitope werden durch Lipidperoxidation gebildet und sind Gefahrensignale, die sowohl im Menschen als auch in Tieren durch natürliche IgM Antikörper, welche von angeborenen B1 Zellen stammen, erkannt werden. Die genaue Rolle von B Zellen und deren Subpopulationen in der Atherogenese ist nachwievor unklar. Vor kurzem wurde jedoch gezeigt, dass die Depletion von B Zellen durch einen CD20 Antikörper, der vorzugsweise B2 Zellen und deren IgG Antikörper jedochnicht B1 Zellen und IgM Antikörperreduziert, die Entstehung von Läsionen drastisch mindert. Auf der anderen Seite weisen Mäuse, die keine Serum IgM Antikörper besitzen, auch verstärkte Läsionsbildung auf. Wir vermuten daher, dass B1 Zellen und die natürlichen Antikörper, die sie freisetzen vor Atherosklerose schützen, während die adaptive Immunabwehr, die von B2 Zellen und ihren IgG Antikörpern übermittelt wird, proatherogen wirkt. Erst kürzlich zeigte eine Publikation, dass B1a Zellen tatsächlich atheroprotektiv wirken, da der Phenotyp von $ApoE^{-/-}$ Mäusen, die nach Entfernung der Milz verstärkt atherosklerotische Läsionen bilden, durch adoptiven Transfer von B1a Zellen gerettet werden konnte. Die Möglichkeit den gesamten B1 Zellpool zu untersuchen wäre durch spezifische Elimination von B2 Zellen gegeben und könnte durch Verwendung eines neutralisierenden Antikörpers, der gegen die BAFF-BAFFR Interaktion gerichtet ist erreicht werden. BAFF-BAFFR Interaktion ist unentbehrlich für das Überleben von B2 Zellen, während BAFF Hemmung in vivo natürliche Antikörper produzierende B1 Zellen unberührt lässt. Diese Diplomarbeit beschäftigt sich primär mit der Funktion des BAFFR auf B1 Zellen und zeigt, dass BAFF-BAFFR Interaktion eine Rolle bei der spontanen IgM Sekretion spielt. Ich

konnte beobachten, dass die Neutralisation des BAFF-Signalweges zu einer erhöhten Produktion von IgM Antikörpern durch B1 Zellen führt. Außerdem konnte ich zeigen, dass Makrophagen die IgM Sekretion von B1 Zellen stimulieren und diese Stimulation direkten Zellkontakt erfordert. Zusätzlich konnte ich bisher noch unbeschriebene BAFFR-Expression auf Makrophagen aufdecken.

ATHEROSCLEROSIS

Atherosclerosis is a chronic inflammatory disorder of large and medium-sized arteries. It is the underlying pathological process of myocardial infarctions and a majority of strokes and the leading cause of death in the world. In atherogenesis many genetic and environmental risk factors, such as hypercholesterolemia, hypertension, diabetes, and smoking, have been identified.(1-3)

The term arteriosclerosis was first introduced by Jean Lobstein in 1829. While for many years it was believed that atherosclerosis develops only through passive accumulation of cholesterol in the vessel wall, it is now well established that both dyslipidemia as well as inflammatory response influence this complex disease process. This is best documented by the fact that increased serum levels of the inflammation marker C-reactive protein have been found to be powerful and independent risk factors for future cardiovascular disease. Moreover, both the innate and the adaptive immune system have been demonstrated to modulate the initiation and progression of atherosclerotic lesions. (4-6)

Atherosclerotic lesion formation:

High circulating levels of cholesterol still represent the most important risk factor for atherogenesis. In the blood a majority of cholesterol is transported by low density lipoproteins (LDL), which are surrounded by a shell of phospholipids, free cholesterol and apolipoprotein B100 (ApoB100). When circulating LDL accumulates in the intima of the artery wall, ApoB100 can bind to proteoglycans on the extracellular matrix. (7) In the artery wall retained LDL particles undergo various modifications including oxidation, resulting in the release of bioactive lipids and the generation of oxidized LDL (OxLDL). The

extent of modification can be minimal, after which the LDL particle can still be bound by the LDL receptors, or massive, in which the particles are not recognized by the LDL receptor but by scavenger receptors (SR) of macrophages and smooth muscle cells. (8) OxLDL carries multiple neo-epitopes that constitute “danger signals” of innate immunity. Various components of innate immunity, including IgM natural antibodies (NAbs, see subsequently), are directed against these danger signals that are also present on apoptotic cells. (9-11)

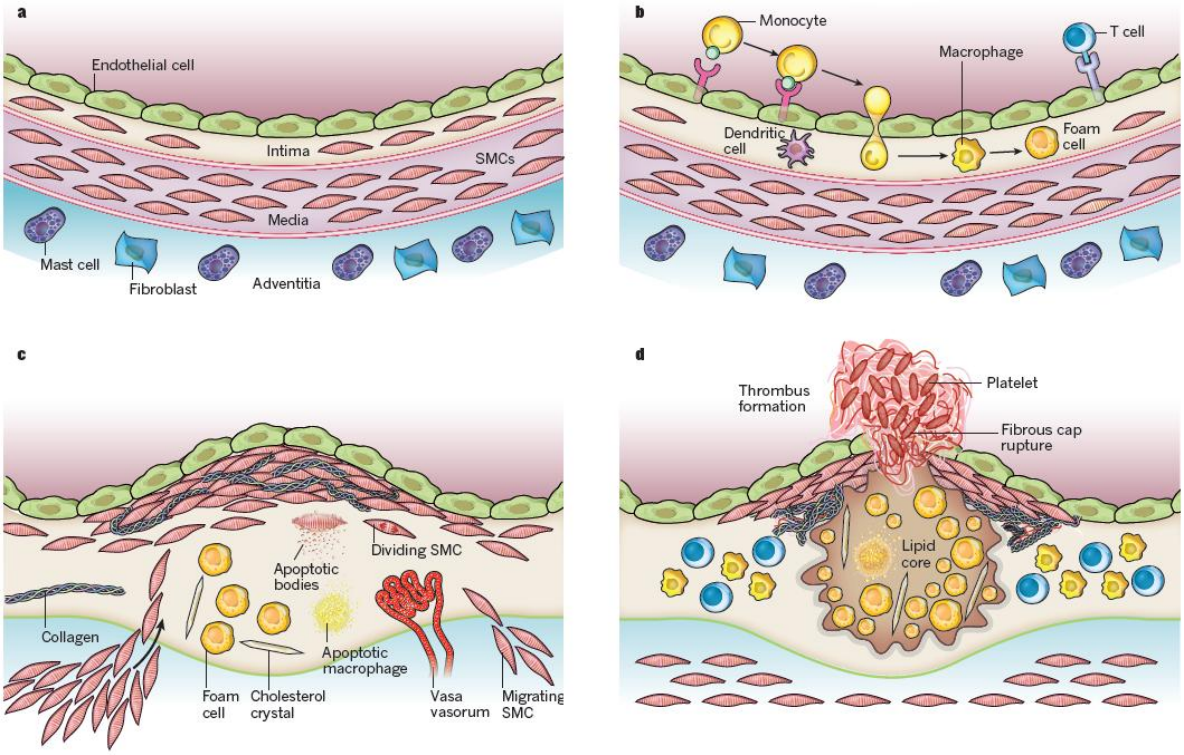


Fig. 1: Stages in the development of atherosclerotic lesions in animal models. (12)

In the initial phase of fatty streak formation a series of stimuli, such as high cholesterol, high blood pressure, or pro-inflammatory mediators activate endothelial cells (EC), which under normal conditions resist the attachment of white blood cells. In turn, EC express adhesion molecules, such as endothelial-cell selectin (E-selectin) and vascular cell adhesion molecule 1 (VCAM-1), and chemokines which mediate the recruitment of monocytes and T cells to the intima. (7, 13) This is a rate limiting step in atherogenesis, as Apo-E-deficient mice ($ApoE^{-/-}$) lacking both E-selectin and platelet selectin (P-selectin) develop less atherosclerosis. (14)

When ECs get activated by OxLDL they also produce macrophage colony-stimulating factor (M-CSF), which stimulates the proliferation of smooth muscle cells and promotes the differentiation of monocytes into macrophages in the intima. (15, 16) Macrophages then up-regulate their scavenger receptors that are involved in the uptake and clearance of modified LDL particles as well as apoptotic cells. (17) This leads to a massive intracellular accumulation of cholesterol esters resulting in the formation of foam cells - hallmark cells of early and late atherosclerotic lesions. (18) This is another key event in atherogenesis, as mice with a mutation in the M-CSF gene, and therefore a nearly complete absence of macrophages, do not develop atherosclerosis when bred to $ApoE^{-/-}$, even though they have elevated circulating cholesterol levels. (16)

The progression to more complex lesions is characterized by immigration of SMC from the medial layer of the artery wall into the subendothelial space. They then proliferate and can also take up modified lipoproteins, further contributing to foam cell formation. Moreover, SMCs synthesize extracellular matrix proteins, including interstitial collagen and elastin that promote the development of the fibrous cap, which covers the plaque. (19) Underneath this cap macrophage-derived foam cells accumulate and undergo apoptosis. Inefficient clearance of dead cells leads to the formation of a lipid-rich acellular core, the so called necrotic core. Degradation and subsequent rupture of the cap releases the pro-thrombotic material from the plaque into the blood and causes thrombus formation and thrombotic occlusion of the artery. (20) (**Fig. 1**)

The role of DCs in atherogenesis might be antigen presentation of modified LDL components in regional lymph nodes. DCs already reside in the artery wall before atherosclerosis development and are thought to promote tolerization to antigens by silencing T cells, but during atherogenesis accumulating danger signals may activate DCs to switch to the activation of adaptive immunity. (18, 21-23)

The impact of T cells on atherosclerosis has been addressed in several studies; they are recruited through similar mechanisms as macrophages, involving adhesion molecules and chemokines. T cells are traditionally divided into Th1 cells, responsible for cell-mediated immunity and secreting IFN-γ and IL-2, and Th2 cells, which secrete IL-4, IL-5, IL-10, IL-13, and provide help for antibody production by B cells. Th1 produce proatherogenic

mediators and contribute to lesion growth and disease aggravation. Regulatory T cells (Treg) inhibit this process. (18, 24, 25)

B cells and the antibodies they secrete do not appear to be required for the development of atherosclerosis but play an important modulatory role, which is discussed in more detail subsequently.

NATURAL ANTIBODIES AND OXIDATION-SPECIFIC EPITOPES:

NAbs are produced very early in life by a specialized subset of B cells, named B1 cells in mice. They are defined as pre-existing antibodies carrying germ line or close to germ line encoded variable regions and are termed natural because they arise spontaneously without antigen exposure. Most NAbs are of the IgM, but to some extent also of the IgG and IgA isotype. (26-29)

The NAb repertoire is thought to be a product of natural selection and has broad specificity for phylogenetically conserved structures of nucleic acids, (glyco)proteins and (phospho)lipids. Numerous foreign and altered self antigens contain those structures and therefore NAbs are involved in the first line defence against invading pathogens. Moreover they recognize altered or stress-induced neo-self-structures on damaged molecules and cellular debris and therefore mediate tissue homeostasis. (26, 27)

Typical examples of stress-induced antigens are lipid-peroxidation derived oxidation-specific epitopes**.** (11) OxLDL, which carries oxidation-specific epitopes, is a highly abundant antigen in atherosclerotic lesions. (30) Recently, it has been shown that oxidation-specific epitopes are targets of NAbs in mice and humans. (31)

Phosphatidylcholine is the major phospholipid of LDL. Peroxidation of phosphatidylcholine leads to the formation of highly reactive breakdown products, such as malondialdehyde (MDA) type adducts, 4-hydroxynonenal (4-HNE), and 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC). (32) IgM and IgG autoantibodies against model epitopes of Ox-LDL, such as Cu2+-oxidized LDL (CuOx-LDL) and MDA-

modified LDL (MDA-LDL), have been documented in the sera and atherosclerotic lesions of animal models and humans. (33, 34) Moreover, hypercholesterolemic rabbits and mice that were actively immunized with homologous CuOx-LDL or MDA-LDL showed robust IgG and IgM responses against the respective antigens and resulted in reduced lesion formation. (35-39)

IgM titers were found to inversely correlate with markers of cardiovascular disease, which suggests that IgM antibodies against epitopes of OxLDL protect from cardiovascular disease in humans. The lowest levels of anti-OxLDL IgM antibodies have been found in patients with the highest risk of developing coronary stenosis and carotid atherosclerosis, respectively. (40, 41)

ApoE$^{-/-}$ mice showed to have very high titers of autoantibodies to epitopes of Ox-LDL. The spleen of those mice was used to generate a set of hybridomas producing IgM antibodies against Ox-LDL. (34) Most of the selected clones bound to either CuOx-LDL or MDA-LDL. One example, the EO6 monoclonal anti-OxPL IgM antibody was found to be structurally and functionally identical to classical natural T15 anti-PC antibodies produced by B1 and marginal zone B cells. (40, 42) Importantly, in terms of atherosclerosis the prototypic anti-Ox-LDL natural IgM antibody T15/EO6 has been shown to reduce lesion formation. (43) Recently, the protective effect of natural IgM Abs was demonstrated by Lewis *et al.* The authors reported that low-density lipoprotein receptor deficient (LDLR$^{-/-}$) mice also deficient in serum IgM antibodies (sIgM$^{-/-}$), and therefore lack NAbs, develop greatly accelerated atherosclerosis in both the aortic root and the entire aorta. (44)

Several explanations for the protective effects of natural IgM in atherosclerosis can be offered: Studies in sIgM$^{-/-}$ mice have shown that NAbs promote the clearance of apoptotic cells. For example, both the OxLDL-specific IgM T15/EO6 and MDA-LDL specific IgM NA17 was able to promote the clearance of apoptotic cells in mice in vivo. (45-47) In addition, NAbs have been shown to protect from autoimmunity – in part by promoting apoptotic cell clearance. Autoimmunity and defective apoptotic cell clearance predispose to increased atherosclerosis, thus the cell clearance property of NAbs may indicate an important atheroprotective function of NAbs.

Furthermore, NAbs have been shown to regulate B cell maturation, maintenance and survival as they can present self antigens to immature B cells. The fact, that in both young and adult $sIgM^{-/-}$ mice B1 cells as well as marginal zone B (MZB)-cells are strongly increased, and B2 cell responses are impaired, illustrates important regulatory functions of natural IgM antibodies. (48-50)

Additionally, B cell receptor (BCR) signaling, which is required for splenic B2 cell selection is also promoted by natural IgM antibodies. Absence or low titers of natural IgM potentially lead to insufficient BCR signaling and autoreactive B cells potentially escape deletion by apoptosis. (51, 52) Eventually, due to their binding properties to altered self structures, NAbs have been demonstrated to play a role in tumor immunosurveillance and neurodegenerative disorders such as Alzheimer's disease. (53-55)

B CELL SUBPOPULATIONS IN ATHEROSCLEROSIS:

During embryonic life B lymphocytes develop in the fetal liver and later on are generated in the bone marrow from hematopoietic stem cells. (56) The maturation process of B cells is divided into different developmental stages that are characterized by the expression of various microRNAs and cell surface markers and the rearrangement of IgH and IgL. (57-59) Following the commitment of the cells to the B cell lineage, they first pass through the pro-B stage, where signaling through interleukin-7 (IL-7) receptor is essential and there is partial rearrangement of IgH. At the pre-B-cell stage, IgH recombination is complete, the cells express a pre-BCR, and IgL chain gene rearrangement begins. (58) The immature B-cell stage is determined by the expression of a mature BCR after successful IgL rearrangements. At this point, immature B cells exit the bone marrow to join the transitional compartment in the spleen (T1 and T2), prior to mature B cells. (60, 61)

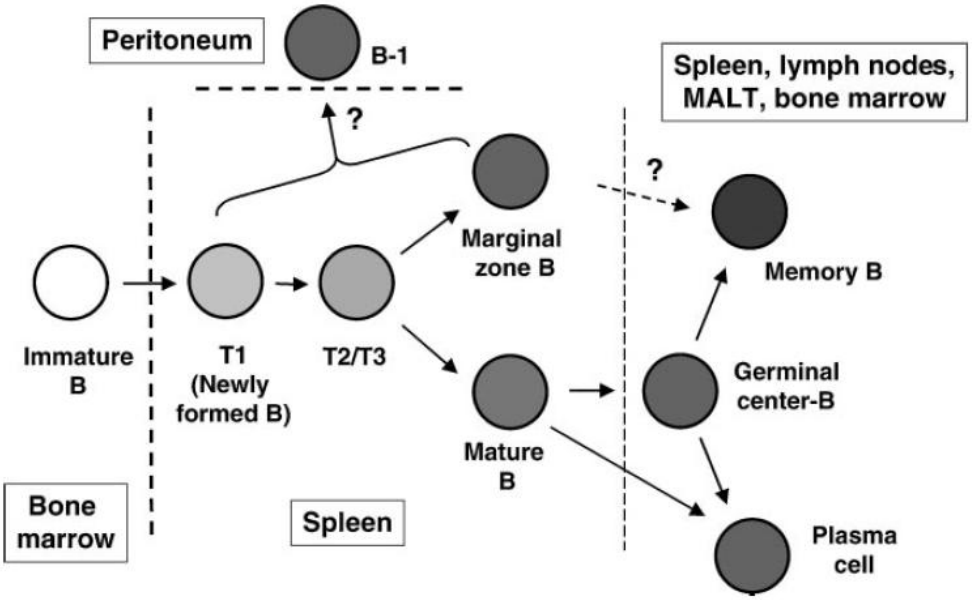


Fig. 2: Stages in B cell maturation. (62)

There are two different subsets of B cells, termed B1 and B2 cells that differ in their surface marker expression, activation requirements and anatomical localization. (63) The B2 lineage includes follicular, germinal center, and plasma B cells. Germinal center formation can occur in the spleen, lymph nodes, or mucosa associated lymphoid tissue and leads to memory and plasma cells. The latter cells can be found in the bone marrow, peripheral tissues, and the spleen. (62) (**Fig. 2**)

B1 cells are the main population in the peritoneal and pleural cavities in mice, but are rare in lymph nodes and have a self-replenishing capacity. (64) The development of B1 cells occurs generally during fetal or perinatal life. There are two different paradigms for the ontogeny of B1 cells. One proposes distinct progenitors for B1 and B2 cells, while the other one suggests their development from a common progenitor, where antigenic selection at the sIgM+ stage decides which way the cell pursues. (65-70)

B cells rarely appear within atherosclerotic plaques, but have been described to be part of tertiary lymphoid organs in the adventitial areas close to plaques. In addition to central and other peripheral lymphoid organs, these B- and T cell clusters may also be sites of antibody production during atherogenesis. (71, 72)

Various studies in atherosclerosis-prone mice provided insights into the important role of lymphocytes in influencing the atherogenic disease process. (73) Studies in $LDLR^{-/-}$ or $ApoE^{-/-}$ deficient mice that were crossed with mice deficient in recombinase activating gene 1 or 2 ($Rag1^{-/-}$ or $Rag2^{-/-}$) showed an overall pro-atherogenic activity of lymphocytes. These functionally T and B cell deficient mice were found to have less severe plaque formation than immunocompetent $LDLR^{-/-}$ and $ApoE^{-/-}$ mice. However, the fact that these mice still develop atherosclerosis led to the assumption that lymphocytes are not necessary for the initiation of atherosclerotic lesion formation. Indeed, in extremely hypercholesterolemic mice, no differences regarding atherosclerosis between Rag deficient and immunocompetent mice could be observed. (74, 75) These data demonstrate that lymphocytes are not required for atherogenesis, but they have the ability to profoundly modulate lesion formation.

The active participation of B cells in murine atherosclerosis could be shown by studies in $ApoE^{-/-}$, which showed aggravated atherosclerosis and reduction of anti-Ox-LDL antibodies, upon splenectomy. Transfusion of splenic B cells, but not T cells, from atherosclerosis-prone $ApoE^{-/-}$ mice into young $ApoE^{-/-}$ mice, and thereby reconstitution of IgM antibodies to Ox-LDL epitopes, resulted in reduced atherosclerosis. (76) Moreover, the consequence of B-cell deficiency was directly tested in lethally irradiated $LDLR^{-/-}$ mice that were reconstituted with bone marrow from µMT mice that lack B cells. The result showed up to 40% increased lesion size accompanied by decreased production of anti-oxLDL antibodies in B-ell deficient mice. (77)

Regarding these studies an overall atheroprotective role for B cells could be suggested, which might be explained by their capacity to secrete protective antibodies and/or to mediate direct immunoregulatory functions. Surprisingly, two different studies in $ApoE^{-/-}$ and $LDLR^{-/-}$ mice that applied CD20 antibody treatment to deplete B cells, showed significantly reduced lesion formation after the treatment. (78, 79) This could be explained by opposing activities of the two major B cell subsets. CD20 Ab treatment showed drastically reduced total IgG and OxLDL-specific IgG titers, whereas the IgM titers against OxLDL were less affected (30% reduction) or even preserved. (79) Consistent with this, natural IgM producing B1 cells in the peritoneum were depleted to a lesser extent than conventional B2 cells, which is consistent with the fact that the peritoneal cavity

represents a protective niche for B1 cells during anti-CD20 treatment. These data suggested a possible pro-atherogenic function of B2 but not B1 cells. Adoptive transfer of splenic B2 cells into total lymphocyte (TKO) or B-cell deficient (µMT ko) Apoe$^{-/-}$ mice indeed resulted in increased atherosclerosis, while adoptive transfer of splenic B1 cells had no effect. (80) Additionally, Kyaw et al. described the B1a subpopulation as atheroprotective in a very recent publication. Removal of the spleen in Apoe-/- mice reduces the peritoneal B1a cell pool and leads to decreased serum IgM titers, while the mice show promoted lesion formation. Adoptive transfer of B1a cells rescues this phenotype and reduces atherosclerosis progression. (81)

Based on these findings, we hypothesize that B cell subpopulations may have contrasting roles in atherosclerosis. B1 cells and the natural antibodies they secrete protect from atherosclerosis, whereas the adaptive humoral immunity mediated through B2 cells and IgG antibodies promote atherogenesis. (78)
A selective depletion of B2 cells by antibodies in an atherosclerosis context has not been the focus of research yet. Neutralizing antibodies, which are directed against the signal molecule B-cell activating fundamental factor (BAFF/BlyS) or one of its receptors (BAFFR) provide the opportunity to obtain the above mentioned effect, which is to achieve B2 cell depletion while preserving the B1 cell-pool in mice. In this context it has already been shown that BAFF-deficient mice have intact B1 cell numbers, whereas the B2 cell numbers are drastically reduced. Additionally, it was shown that BAFF inhibition in vivo leaves natural antibody producing cells unaffected. (82, 83)

B-CELL ACTIVATING FACTOR SIGNALING:

BAFF and APRIL

B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) are tumor necrosis factor (TNF) family members. They are mainly produced by innate immune cells

such as neutrophils, macrophages, monocytes, DCs and follicular DCs (FDCs) and are crucial for peripheral B cell survival. Type I interferons (IFNs), IFNγ, IL-10 and granulocyte colony-stimulating factor (G-CSF), as well as the activation of Toll-like receptors, such as TLR4 and TLR9, increase the expression of these two ligands. (62, 84, 85) Moreover, T cells, activated B cells and B cell chronic lymphocytic leukemia (BCLL) cells can also produce the homotrimeric type II transmembrane proteins BAFF and APRIL. (85)

BAFF is initially synthesized as cell-bound protein and following activation of the cell proteolytically cleaved at furin consensus sequences yielding a soluble cytokine. While APRIL does not exist in a membrane bound form, as the biologically active protein is directly secreted after intracellular processing in the Golgi, BAFF also appears membrane bound. (86, 87) Released BAFF usually forms trimers, but it can also further assemble to BAFF 60-mers, with still accessible receptor binding sites. (88) Furthermore, BAFF and APRIL can form heteromers. (87)

Receptors

Common receptors for BAFF and APRIL are B cell maturation antigen (BCMA, TNFRSF17) and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI, TNFRSF13B), but BAFF also binds to the BAFF receptor (BAFFR, BR3, TNFRSF13C). (87, 89)

The receptors are mainly expressed by B cells. (90, 91) The expression of BAFFR is low on newly formed immature murine B cells, but increases as these cells evolve and is ultimately expressed by all mature B cells. (90) Furthermore, the BAFFR is also expressed by regulatory T cells. (92) TACI is produced by all peripheral B cells, marginal zone B cells and B1 B cells. (93) Signaling through TACI and BAFFR have opposite roles. While BAFFR transmits survival signals and positively regulates B-cell homeostasis, signaling through TACI reduces B cell numbers. (93, 94)

When BAFF ligand binds to the BAFFR, at least a trimer has to bind to initiate signalling, as three independent receptors need to be bound by the ligands to arrange in close

proximity for signal transmission. For signalling through TACI binding of BAFF 60-mers are necessary. (88)

TNF receptor-associated factors (TRAFs) are trimeric intracellular proteins that bind linear TRAF-binding sequences present in several receptors including the BAFFR. The affinity of TRAFs for monomeric receptor peptides is low, but ligand-induced oligomerization stabilizes the interaction. (95) BAFFR recruits exclusively TRAF3, which usually interacts with NF-κB-inducing kinase (NIK), the upstream kinase responsible for NF-κB2 processing. Together with TRAF2 it induces NIK degradation by the proteasome; thus preventing the activation of the alternative NF-κB pathway. When BAFF is bound to the BAFFR, TRAF3 is recruited and degraded in a TRAF2-dependent manner, resulting in the stabilization of NIK and activation of NF-κB2, which then promotes B cell survival. (59, 96-98) (**Fig. 3**)

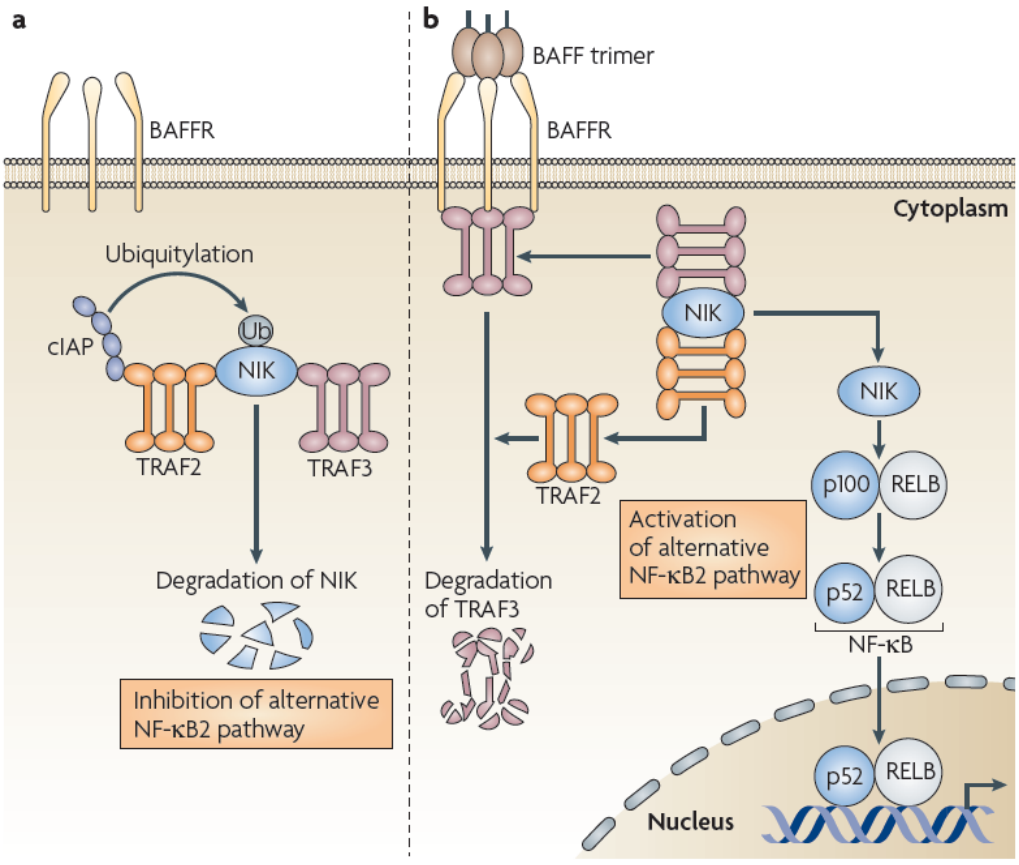


Fig. 3: BAFF-BAFFR signaling. (85)

The expression of a functional BCR is one essential event for early B cell development and later peripheral B cell homeostasis. However, BAFF plays an additional factor in the

persistence of peripheral B cells. The first indication that BAFF may be a survival factor came from the expanded peripheral B cell numbers and elevated serum Ig levels, found in mice over expressing BAFF. (59, 99, 100)

Studies in mice have shown that BAFF is not playing a critical role in B cell development in the bone marrow. Moreover the survival of immature or splenic T1 cells is not dependent on BAFF signaling. (61, 83, 101, 102) Nevertheless, a fundamental role for BAFF in the survival of splenic T2 B cells, and mature and marginal zone (MZ) B cells could be indicated. This was supported by analysis of BAFF-treated and BAFF-transgenic mice, which showed normal bone marrow and T1 compartments, but expanded T2 and MZ populations. (103, 104) In contrast, BAFF or APRIL are not necessary for the survival of B1 B cells, memory B cells and a small population of mature splenic B cells. (102, 105, 106) Sasaki et al. generated mice deficient in the BAFFR that showed a similar phenotype as BAFF deficient mice: B cells were found to have a developmental block at the transitional stage T1 to T2, and a 40-fold reduction in numbers of mature B2 cells with very few - if any - MZ B cells, while the B1 compartments stayed unaffected. Whether BAFF is also crucial for the survival of mature B cells in vivo was addressed by Rauch et al. when they generated an anti-BAFF-R monoclonal antibody and studied its effect in mice. Injections of a blocking anti-BAFF-R antibody led to dramatic depletion of the mature B cell compartment, and showed that almost all follicular and half of the marginal zone B cells are dependent on BAFF-BAFFR signalling. (102, 107)

A correlation between excess BAFF and autoimmunity could be shown in the BAFF-transgenic mouse. This mouse further demonstrated the first animal model of T cell-independent lupus. (108) Elevated BAFF levels also have been found in sera of patients with various autoimmune conditions. (109) Moreover, BAFF expression has been detected in non-lymphoid breast cancer cells from epithelial origin and increased levels of BAFF have been shown during inflammation, allergy and viral infection, possibly as a consequence of type I IFNs production, which are known to promote BAFF production. (85, 110) The fact that increased BAFF serum levels have been found in different diseases triggered the development of BAFF an APRIL antagonists. Human Genome Sciences (HGS)

and GlaxoSmithKline (GSK) developed a fully human BAFF-specific monoclonal antibody (Belimumab) that has been tested successfully in clinical trials. It has been approved by the Food and Drug Administration (FDA) in March 2011, for the treatment of adult patients with active, autoantibody-positive systemic lupus erythematosus (SLE).

AIM OF THESIS

Selective depletion of B2 cells while preserving the B1 cell pool in atherosclerotic mouse models would be an ideal way to strengthen the hypothesis that B1 cells and the natural antibodies they produce protect from atherosclerosis, whereas the adaptive humoral immunity mediated through B2 cells and IgG antibodies promote atherogenesis. (78) For this purpose we believe that neutralizing antibodies, which are directed against the signal molecule B-cell activating fundamental factor (BAFF/BlyS) or one of its receptors (BAFFR) provide the opportunity to obtain the above mentioned effect. It has been described that B2 cell survival is dependent on BAFF-BAFFR signaling, while for B1 cells it is not a determinant for survival. In this context it was shown already that BAFF-deficient mice have intact B1 cell numbers, whereas the B2 cell numbers are drastically reduced. (83) Rauch et al. showed that injection of a blocking anti-BAFFR mAb (9B9 Hybridoma) that prevents BAFF from binding showed drastically decreased follicular and marginal zone B cell numbers after 2 weeks, whereas B1 cells were not affected. This study established a central role for BAFF-BAFFR signaling in the in vivo survival of both follicular and marginal zone B cell pools. Moreover using this antibody they showed BAFFR expression on all mature B cells, namely B2, MZB, B1 (B1a and B1b) as well as on the three immature transitional splenic B cell subsets. (102)

The central experimental aim of this thesis is to understand the importance of BAFF signaling through BAFFR in B1 cells with respect to the production of atheroprotective NAbs. Using a blocking antibody for BAFFR, I addressed the role of the BAFF-BAFFR interaction on B1 cells in vitro with respect to their capacity to secrete natural IgM antibodies.

BAFFR blocking in total peritoneal cells:

The highest number of B1 cells can be found in the peritoneal cavity. Thus, I first investigated a possible effect of BAFF-BAFFR interaction blockage by examining the role of the receptor in total peritoneal cells. I first tested whether blocking the receptor with anti-BAFFR antibody, which was kindly provided by Dr. Rolink (University of Basel) (102), can influence spontaneous IgM secretion.

Total peritoneal cells were isolated from C57BL/6 mice by peritoneal lavage, cultured in vitro, and supernatants were analyzed for IgM secretion. I compared IgM secretion of these cells over 48h under three different conditions: 1) cells left untreated; 2) cells treated with the anti-BAFFR blocking antibody; and 3) cells in presence of an isotype matched IgG2b control antibody. IgM antibody concentrations in the supernatants were measured by a sandwich ELISA. The results of this measurement (**Fig.4-a**) showed that IgM concentrations are increased when cells are incubated in presence of anti-BAFFR compared to untreated peritoneal cells and those treated with the isotype control. Moreover, the increased IgM secretion in the aBAFFR treated cells was dose-dependent, as the IgM concentrations in the supernatants increased with the amount of antibody provided. This experiment was performed several times in biological replicates and different incubation times ranging from 24h to 120h, with similar results. **Figure 4-c** presents the representative results of IgM measurement after 72h and 96h, respectively.

Additionally I examined the binding of the aBAFFR that was commercially purified from 9B9 hybridomas (102) by BIOTEM through Western blot. The BAFFR contains 175 amino acids, has a size of about 19kDa and can be found in many glycosylated forms. Using the aBAFFR antibody as detection antibody for the blot, I could identify a specific band in

lysates of C57BL/6 splenocytes as well as of the B1 cell line BCL-1 that was used as control (**Fig.4-b**).

Exclusion of endotoxin side effects:

Because endotoxin has been shown to stimulate IgM secretion, a potential contamination of the antibody with LPS needed to be excluded. Endotoxin measurements in the aBAFFR antibody aliquots showed only negligible values of 0,00368 ng per µg of protein in one aliquot and 0,000234 ng per µg of protein in the second aliquot. The control antibody contained only 0,000117ng endotoxin per µg of protein. To further confirm that the increased IgM production was really due to specific BAFFR blocking and not a result of endotoxin contamination I repeated the same assay that is presented in Fig.4-a in the presence of the LPS-neutralizing agent Polymyxin B (PMB).

PMB is used to absorb LPS in reagents that are used for immunological experiments, as LPS can induce strong immune reactions, which can interfere with the experimental results. Consistent with the low levels of endotoxin in the antibody preparations, addition of PMB did not decrease the IgM production induced by BAFFR-neutralization after 48h (**Fig.4-d**). This supports that the effect we see in the increase of IgM secretion is obtained by interaction of the receptor with the blocking aBAFFR antibody.

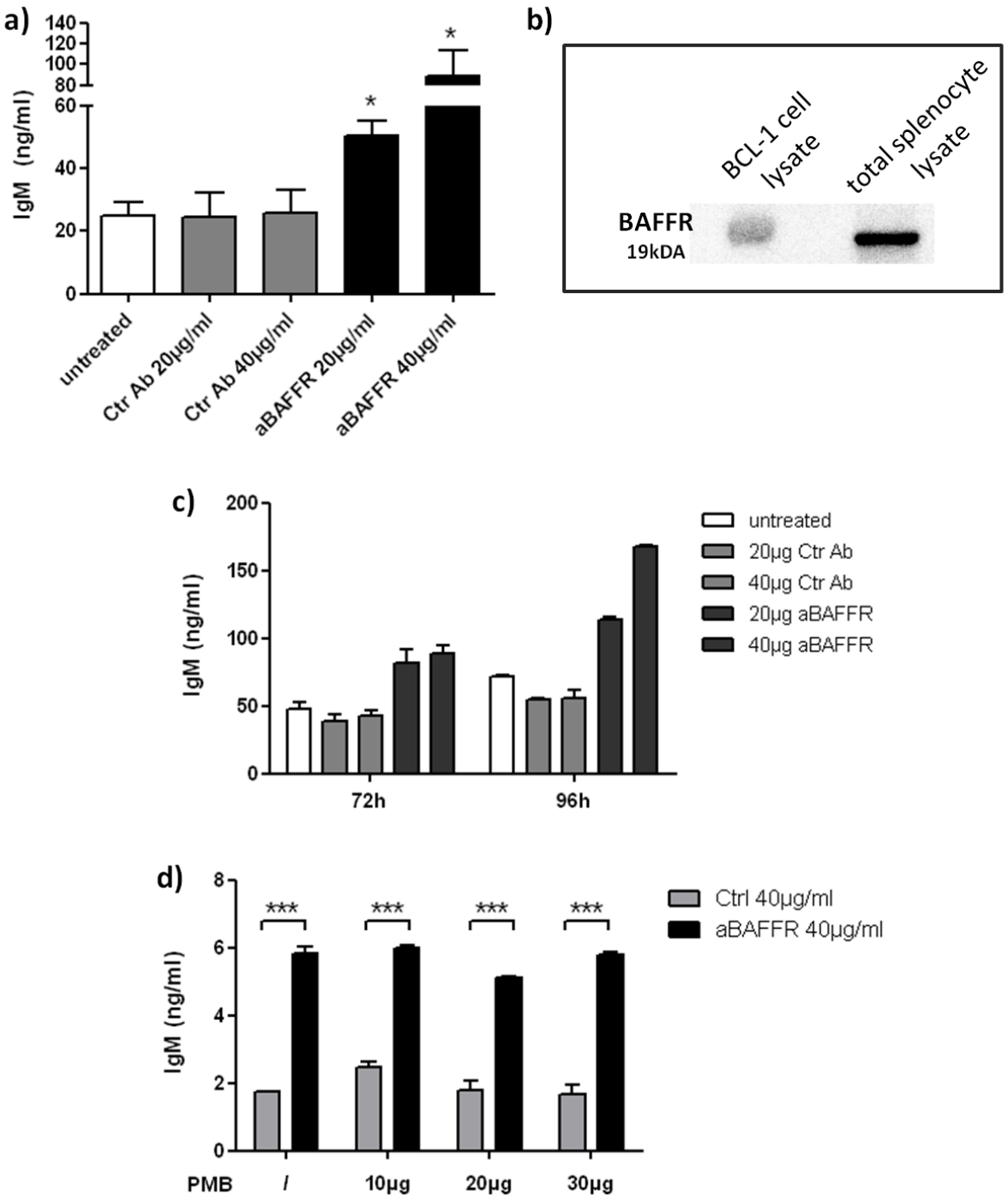


Fig. 4: a) IgM secretion after aBAFFR treatment of mouse peritoneal cells. After 48h incubation supernatants of aBAFFR treated cells, in both concentrations 20µg/ml (50,3ng/ml) and 40µg/ml (88,3ng/ml), show significantly increased IgM concentrations compared to untreated (24,7ng/ml) and 20µg/ml (24,6ng/ml) or 40µg/ml (25,8ng/ml) control antibody treated cells. Data are presented as mean ± SEM of 3 replicates; * indicates $p<0,05$ versus untreated and control antibody. Data are representative of at least two independent experiments. **b) Western blot for splenocyte- and BCL-1 cell-lysate**. Specific binding of aBAFFR Ab to proteins with a molecular

weight of the BAFFR (19kDA) is displayed. **c) IgM secretion after aBAFFR treatment of mouse peritoneal cells.** After 72h aBAFFR treated cells, in both concentrations 20µg/ml (81,8ng/ml) and 40µg/ml (88,9ng/ml), show increased IgM concentrations compared to untreated (47,8ng/ml) and 20µg/ml (38,9ng/ml) or 40µg/ml (42,3ng/ml) control antibody treated cells. After 96h aBAFFR treated cells, in both concentrations 20µg/ml (113,3ng/ml) and 40µg/ml (167,3ng/ml), show significantly increased IgM concentrations compared to untreated (71,2ng/ml) and 20µg/ml (54,9ng/ml) or 40µg/ml (55,9ng/ml) control antibody treated cells. Data are presented as mean ± SEM of 2 replicates. **d) Exclusion of endotoxin contamination**. IgM titers in supernatants of aBAFFR treated cells are higher than those of the control antibody treated cells. PMB at different concentrations did not neutralize the effect. Data are presented as mean ± SEM of 3 replicates, *** indicates p<0,0001. Data are representative of at least two independent experiments.

aBAFFR treatment proliferation assay

To test whether the increase in IgM after aBAFFR treatment might be due to increased proliferation of B1 cells during the treatment I performed a proliferation assay. I cultured total peritoneal cells for 48h alone or in the presence of aBAFFR antibody or control antibody. The percentages of the major cell populations present in the different wells were then analyzed by FACS staining.

The total number of cells acquired at the end of the experiment was not different between the conditions. After gating on living cells, there was no difference in percentages between B1, B2, macrophages and T cells with and without treatment with aBAFFR antibody (**Table 1**). This suggests that blocking of the receptor does not lead to proliferation of B1 or B2 cells and therefore proliferation cannot explain the increase in IgM levels.

% of viable cells ±SD	**untreated**	**control antibody**	**anti-BAFFR antibody**
B1 cells	55 ±0,28	53,63 ±0,21	56,37 ±1,10
B2 cells	12,43 ±0,47	10,50 ±0,26	10,08 ±0,32
Macrophages	15 ±0,71	15,17 ±1,11	14,4 ±1,61
T cells	12,8 ±0,99	11,9 ±0,72	11,93 ±0,47

Table 1: Percentages of different cell populations present after antibody treatment in vitro. Gated on viable cells the percentages of B1, B2, macrophages and T cells stay unchanged after treatment with aBAFFR, compared to control antibody and untreated cells. Data are representative of at least two independent experiments.

aBAFFR effects equal aBAFF treatment effect in peritoneal cells

Treatment of total peritoneal cells with aBAFFR antibody resulted in an increase in IgM secretion. To support this finding, I decided to interfere with the BAFF-BAFFR signaling by using an antibody against BAFF, the only ligand for the BAFFR. The hamster-anti- BAFF monoclonal antibody used was provided by GSK as part of collaboration to investigate its role in atherosclerosis.

Again peritoneal cells were isolated from C57BL/6 mice and cultured in the presence of aBAFFR or aBAFF antibodies, respectively. Cells were incubated for 72h and supernatants analyzed for the presence of IgM. Both blocking the receptor and neutralizing its ligand with aBAFF resulted in increased spontaneous IgM secretion (**Fig.5**).

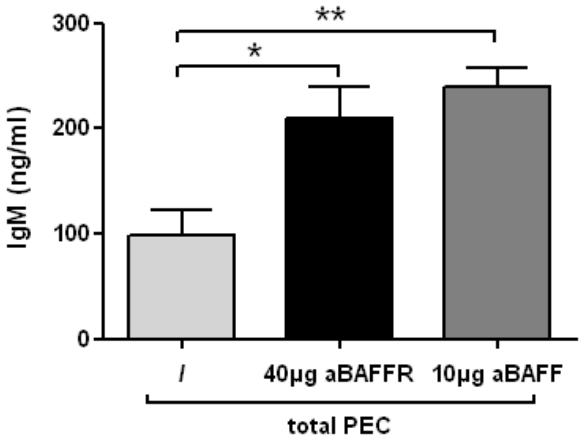


Fig. 5: **Comparison of the aBAFFR and aBAFF effect on IgM production in peritoneal cells.** Both aBAFFR (210,2ng/ml) and aBAFF (240,2ng/ml) treated peritoneal cells showed significantly increased IgM secretion after 72h compared to untreated cells (98,2ng/ml). The difference in IgM

secretion between aBAFFR and aBAFF treated cells was not significant. Data are presented as mean ± SEM of 4 replicates, * indicates p<0,05, ** indicates p<0,005.

BAFFR expression on cells in the peritoneum

The expression of BAFFR on the surface of peritoneal cells was investigated. Previous publications reported that B cells and activated T cells, in contrast to non-activated T cells, express BAFFR. (90-92, 111) There were no indications for macrophages expressing BAFFR in the literature. For this purpose I examined the surface expression on peritoneal cells by flow cytometry using a FITC-labeled aBAFFR antibody.

Total peritoneal cells were isolated from C57BL/6 mice, and cells were stained with antibodies against B220, which is a B cell marker, CD11b to distinguish between B1 and B2 cells and macrophages, CD5 to distinguish between B2 cells and T cells, and BAFFR to examine receptor expression on the cell surface. I could confirm the expression of the BAFFR on B cells (B1a, B1b and B2), while T cells were negative for it. Surprisingly, macrophages also exhibited positive BAFFR staining indicating surface expression of the receptor on peritoneal macrophages (**Fig.6-a**). To confirm this finding, I purified each peritoneal cell population by FACSorting to obtain B1 cells, macrophages and T cells. The purity of each population was greater than 80%. Isolated cells were then stained with the FITC-conjugated aBAFFR antibody to study BAFFR expression. As indicated in **Fig 6-b**, positive BAFFR staining was found on B1 cells as well as macrophages, whereas T cells were negative as seen before.

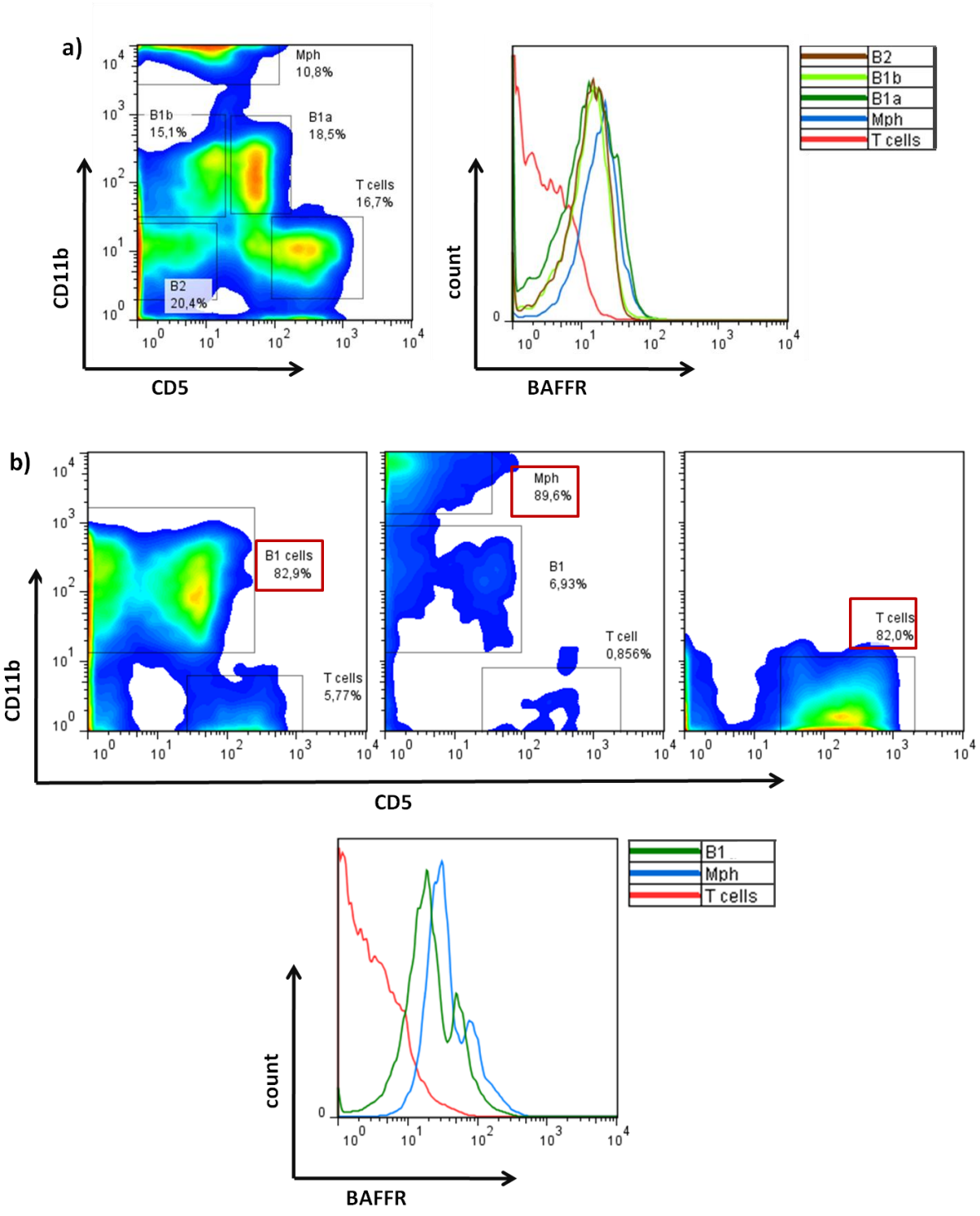


Fig. 6: FACS-Staining for BAFFR on peritoneal cells. a) Gated on viable cells, individual cell populations are shown based on CD11b and CD5 expression. The histogram plot on the right shows BAFFR expression intensity for each cell population. B1a, B1b, B2 and macrophages are positive for BAFFR staining, while T cells appear negative. **b)** After FACSorting following purities were achieved: B1 cells – 82,9%, macrophages – 89,8%, T cells – 82,0%. B1 cells were slightly contaminated with T cells and some B1 cells could be observed in the macrophages enrichment.

The T cell pool contained few B2 cells. Further staining for BAFFR showed that T cells do not express the BAFFR and therefore show no positive signal in the FITC channel. B1 cells and macrophages both give a positive signal which indicates that they express the receptor. Data are representative of at least two independent experiments

BAFFR Expression on RAW and BCL-1 cells

After observing BAFFR expression on peritoneal macrophages, I additionally investigated its expression in the RAW macrophage cell line. I stained both RAW cells as well as BCL-1 cells, which served as positive control. The obtained results indicated once more BAFFR expression on macrophages as signal was almost as strong as that obtained with BCL-1 cells. Stained BCL-1 and RAW were compared to unstained cells (**Fig.7**).

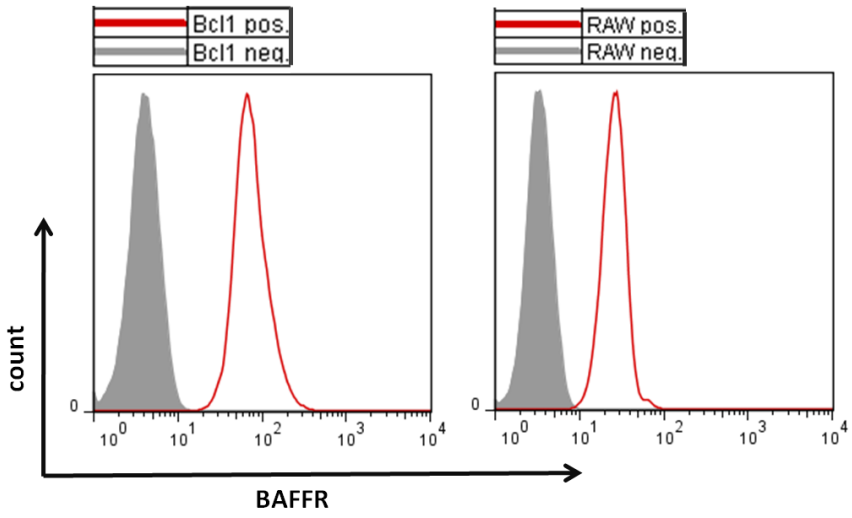


Fig. 7: FACS-Staining for BAFFR on RAW and BCL-1 cells. BCL-1 cells and RAW macrophages give a distinct positive signal, indicating express of the BAFFR. Negative controls were unstained cells of each cell line.

XBP-1 and Blimp-1 expression in peritoneal cells after aBAFFR treatment

B-lymphocyte-induced maturation protein 1 (Blimp-1) has an essential role for antibody secretion by B2 and B1 cells, ex vivo and in vivo, and it is known to be involved in IgM secretion. Blimp-1 is necessary for full induction of IgH, J-chain, and X-box binding protein 1 (XBP-1) mRNAs in B2 cells. B1 cells, like B2 cells, require Blimp-1 and Blimp-1–dependent de-repression of XBP-1 to secrete Ig. (112, 113)

I examined if the expression of these two genes in total peritoneal cells is changed following aBAFFR treatment, as IgM secretion is induced. Total peritoneal cells were incubated with aBAFFR or isotype control antibody for 4h, 24h and 48h. IgM titers were measured in the supernatants to confirm the increase in the supernatants of aBAFFR treated cells after 24h and 48h. Cells were lysed, mRNA isolated and a quantitative RT-PCR was performed for XBP-1 and Blimp-1. Gene expression was normalized to Cyclic B. No difference in gene expression levels of the two target genes could be identified (**Fig.8**).

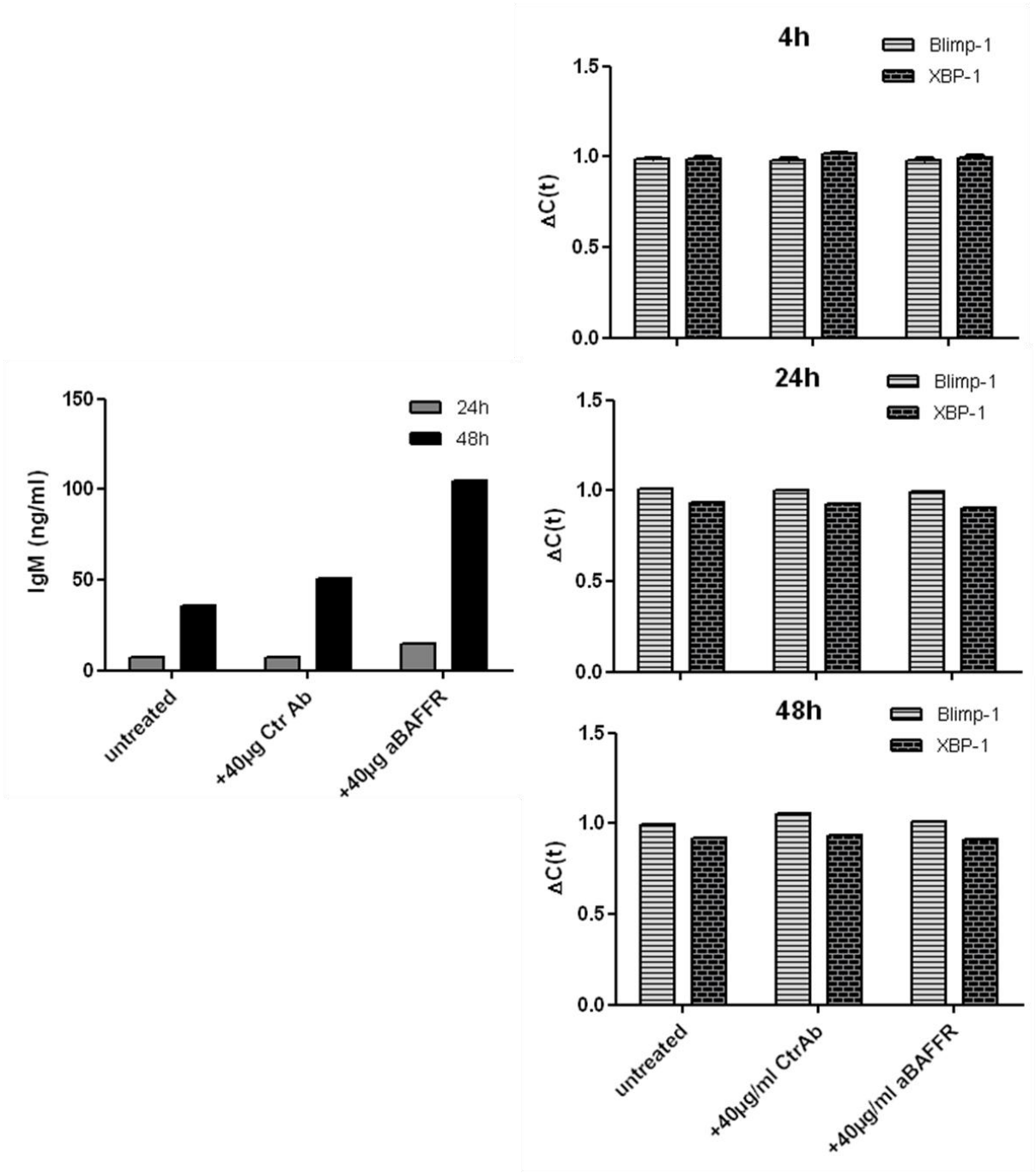


Fig. 8: RT-PCR for XBP-1 and Blimp-1 in aBAFFR treated peritoneal cells. IgM ELISA shows increase in IgM levels after 24h and 48h, when cells are treated with aBAFFR antibody (left). qPCR shows no difference in gene expression of XBP-1 and Blimp-1 (right).

Serum BAFF levels in LDLR$^{-/-}$ mice on ND or HFD

Previous work in our group showed that LDLR$^{-/-}$ mice that were fed a high fat diet for 12 weeks (HFD, 0.2% Cholesterol, 21% milk fat; TD88137 Sniff) show dramatically increased IgM titers compared to mice fed a regular chow diet (ND). In the foregone data I presented how the BAFF-BAFFR system could modulate IgM secretion. Therefore, I wanted to investigate if there may be a difference in serum BAFF levels between the LDLR$^{-/-}$ mice on HFD and those on ND. BAFF levels in serum were measured using a commercial ELISA. The results of the BAFF measurements show that there is no difference in serum BAFF concentrations between mice on ND and mice on HFD (**Fig.9**).

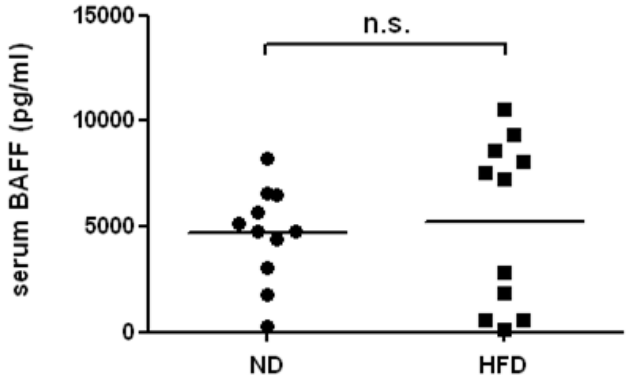


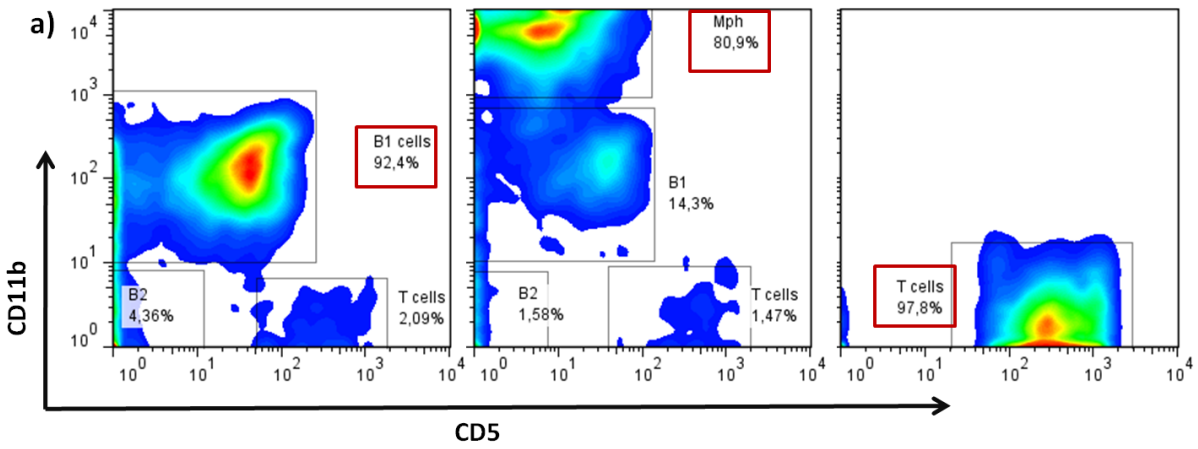
Fig. 9: Serum BAFF titers of LDL$^{-/-}$ mice after 12 weeks on ND or HFD. Sera of 11 mice per group were analyzed and no significant differences in BAFF levels were found between the two groups. In the ND mice the average serum BAFF concentration was 4663 pg/ml and in HFD mice the average was 5209/ml. Each data points indicate the levels obtained from individual mice, and the horizontal line indicates mean value for each group; n.s. indicates p>0,05 using unpaired t-test.

aBAFFR treatment of sorted peritoneal B1 cells :

The observed effects of aBAFFR on IgM secretion may come from direct interaction of the aBAFFR antibody with the BAFFR on B cells, thereby stimulating more secretion, or

through blockage of the receptor resulting in increased availability of BAFF ligand to bind to other receptors, such as TACI, leading to more IgM secretion. I examined this by either only blocking the receptor on sorted B cells or by additionally providing cells with excess of recombinant BAFF protein. If only blocking of the receptor on B1 cells was sufficient, increased IgM titers in the supernatants of cells treated with the aBAFFR antibody should be observed. If binding to TACI was involved IgM levels are expected to increase, when excess BAFF protein is present independent of BAFFR blockage, as the ligand would have the capacity to bind to TACI.

For this purpose total peritoneal cells were isolated from C57BL/6 mice, and B1 cells, macrophages, and T cells were sorted subsequently. Purified B1 cells were treated accordingly in vitro for 72h. Recombinant BAFF protein did not stimulate IgM secretion, whereas blocking of the BAFFR increases IgM titers significantly independent of presence or absence of recombinant BAFF protein.



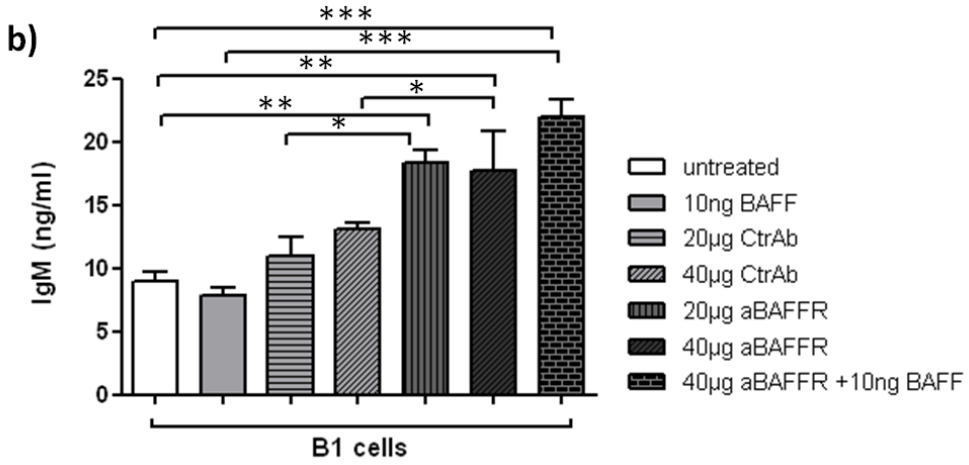


Fig. 10: Antibody treatment of sorted peritoneal B1 cells. a) Fow cytometry plots of sorted cells indicating purities of cell populations obtained. B1 cells were 92% pure, macrophages 81% and were contaminated mainly with B1 cells, T cells were 98% pure. **b)** IgM levels after B1 cells were treated with different antibodies. Increase in IgM could be shown when cells were incubated with different concentrations of aBAFFR antibody (18,3ng/ml and 17,7ng/ml) and also when cells were pre-treated with aBAFFR and then provided with recombinant BAFF protein (21,9ng/ml) compared to untreated cells (9,0ng/ml) and control Ab (10,9ng/ml and 13,0ng/ml) treated cells. Recombinant BAFF (7,88ng/ml) alone did not cause change in IgM secretion. Data are presented as mean ± SEM of 3 replicates, * indicates p<0,05, ** indicates p<0,005, *** indicates p<0,0001. Data are representative of at least two independent experiments.

Macrophages stimulate IgM secretion in primary sorted B cells

Following peritoneal cell sorting (see purity **Fig. 10-a**) I also investigated whether the presence of other cell types together with B1 cells in culture modulates IgM secretion independently of the BAFFR signaling. Therefore, I combined the major cell types present in the peritoneal cavity to see if they modulate the IgM secretion capacity of B1 cells. Sorted B1 cells were cultured either alone or in the presence of macrophages or T cells or both. Interestingly, the presence of macrophages resulted in an 18-fold increase of IgM secretion during 72h compared to B1 cells alone. The presence of T cells on the other hand had no effect. When all three populations were present B1 cells produced more IgM compared to B1 cells alone, but only half of the concentrations that were produced when B1 cells and macrophages were in culture in the absence of T cells (**Fig. 11**).

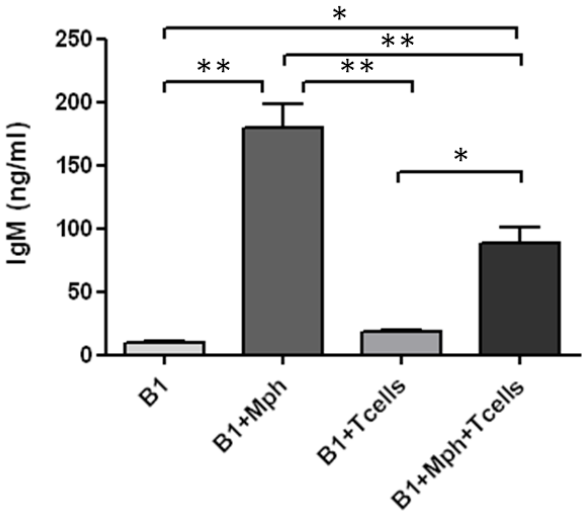


Fig. 11: IgM titers in co-cultures of primary sorted cells. In the supernatants where only B1 cells are present the mean IgM concentration found is 9,3ng/ml, when macrophages are present it increases to 179,4ng/ml, when T cells are present it is 18,2ng/ml and when all three populations are together it is 887ng/ml. Data are presented as mean ± SEM of 3 replicates, * indicates $p<0,05$, ** indicates $p<0,005$.

Cell contact mediated stimulation of IgM secretion by macrophages

Taking the above mentioned results into consideration the question arose, whether the potential of macrophages to stimulate B1 cells to secrete IgM was dependent on cell contact or on the secretion of soluble factors, which then interact with B cells. To test this I used a transwell culture system in which the two cell populations are separated by a membrane allowing only small molecules like cytokines in medium to pass (**Fig. 12**).

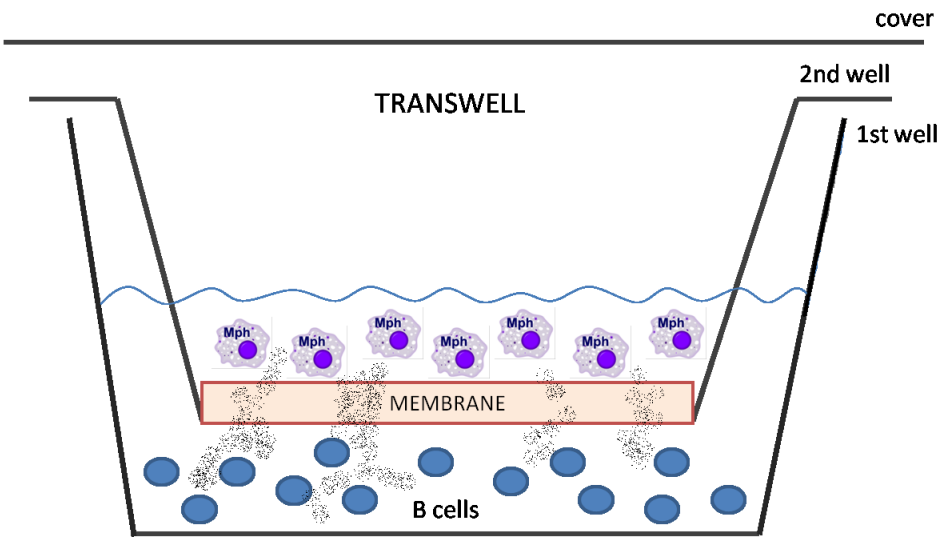


Fig. 12: Schematic representation of the transwell-system. B cells were placed in the lower well and macrophages in the 2nd well on top of the membrane. Exchange of soluble factors is possible but cell contact of the two cell types is not.

Sorted B1 cells (purity **Fig. 10-a**) were placed in the lower part of the transwell-plate and macrophages were placed in the upper well. In parallel experiments B1 cells were directly co-cultured with two different concentrations of macrophages. When macrophages were directly present in the B1 cell culture, IgM production was strongly induced and dependent on the number of macrophages. In contrast, supernatants obtained from the transwell assay did not show an increase in IgM production compared to B1 cells alone. These data suggests that direct cell-cell contact is required for the stimulation of IgM secretion by B1 cells through macrophages. (**Fig. 13**)

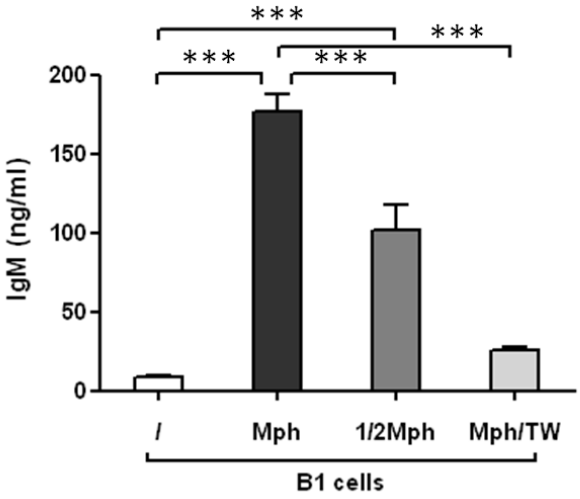


Fig. 13: IgM secretion in co-culture and transwell assay of primary sorted B1 cells and macrophages. Strong increase in IgM titers can be seen when B1 cells are cultured with Mph (179,4ng/ml) and the titers decrease with the decrease in macrophages cell numbers present (101,2ng/ml). The transwell (TW, 25,8ng/ml) supernatant measurement shows no significant difference compared to B1 cells alone (9,3ng/ml) in culture. Data are presented as mean ± SEM of 3 replicates, *** indicates p<0,0001. Data are representative of at least two independent experiments.

RAW cells induce cell contact mediated IgM secretion in BCL-1 cells

To strengthen the finding of the capacity of macrophages to stimulate IgM secretion by B1 cells, I did similar assays using BCL-1 cells, a B cell lymphoma cell line and RAW macrophages. In the experimental approach, I evaluated whether increasing numbers of RAW macrophages increase IgM secretion by BCL-1 cells. For this, 50,000 BCL-1 cells per well were cultured for 10h with increasing numbers of RAW macrophages (between 0 and 150,000 cells per well). Indeed, increased numbers of RAW cells resulted in increased production of IgM by BCL-1 cells, which strongly supports the capacity of macrophages to up-regulate IgM secretion by B1 cells (**Fig. 14-a**).

Moreover, I further validated the importance of cell-cell contact for this activity of macrophages using these two cell lines. Again I used the transwell system to test this. BCL-1 cells were plated either alone, together with RAW cells, or separated from RAW cells by a membrane, and incubated for 18h in the transwell culture and supernatants were analyzed for IgM secretion. Consistent with the results obtained with primary cells, the results using RAW cells and BCL-1 cells demonstrate that cell-cell contact is required, as only when BCL-1 cells and RAW cells were cultured together an increase in IgM production was observed (**Fig. 14-b**).

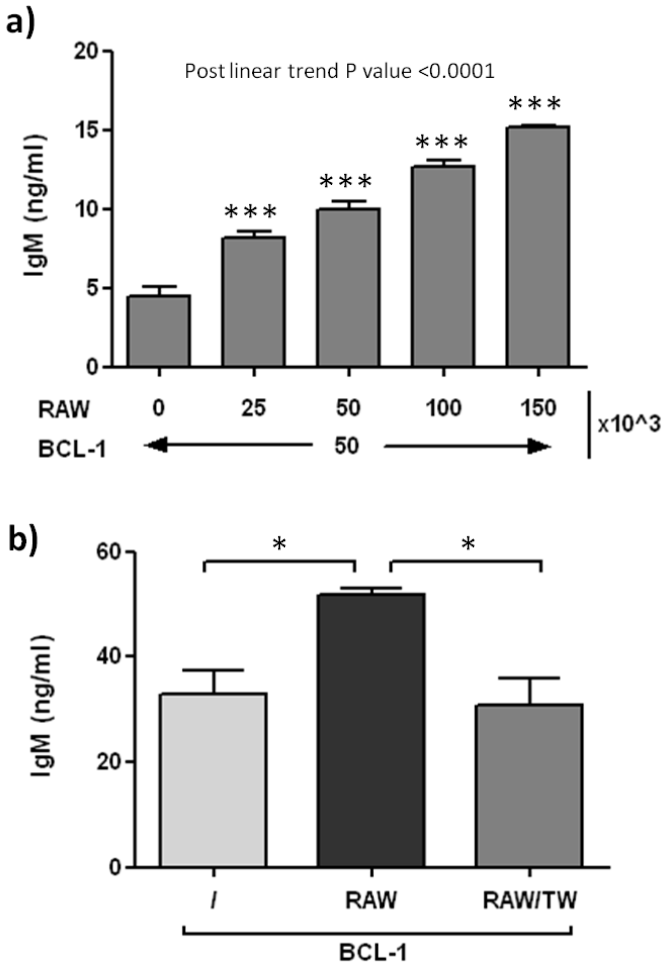


Fig. 14: IgM secretion in BCL-1 and RAW co-cultures. a) BCL-1 cells cultured with different numbers of RAW macrophages. The graph shows IgM levels in supernatants of cultures with a fixed BCL-1 cell number of 50,000 cells per well and increasing RAW cell numbers ranging from 0 to 150,000 cells per well. IgM titers increased with numbers of RAW cells. Data are presented as

mean ± SEM of 2 replicates. Data are representative of at least two independent experiments. **b)** Transwell assay with BCL-1 and RAW cells. BCL-1 cells and RAW cells were plated in transwell assays, separated by a membrane, or cultured in regular 96-well plates. BCL-1 cells (33,1ng/ml) and BCL-1 /RAW transwell supernatants (30,9ng/ml) were found to have equal IgM titers while in the regular BCL-1 /RAW co-culture titers were again significantly increased (51,9ng/ml) compared to BCL-1 cells alone or BCL-1 cells cultured in the transwell system. Data are presented as mean ± SEM of 3 replicates, * indicates $p<0,05$. Data are representative of at least two independent experiments.

IgM stimulation capacity of macrophages from LDLR$^{-/-}$ mice on ND or HFD

It has been shown that atherosclerotic mice on a high cholesterol diet have higher IgM titers. Thus, I hypothesized that this could be due to an increased capacity of macrophages from cholesterol-fed mice to stimulate IgM secretion of B1 cells. To test this I fed one group of LDLR$^{-/-}$ mice a HFD for 12 weeks. The other group received a ND. Following sacrifice, macrophages were isolated from the two groups of mice by FACSorting of peritoneal cells obtained by lavage. In parallel, B1 cells were obtained from the peritoneal cavities of naïve C57BL/6 mice by FACSsorting (sorting purity **Fig. 15-a**). B1 cells were then co-cultured with either macrophages obtained from mice on ND or HFD, respectively. After 72h IgM levels in supernatants were assessed by ELISA. Both types of macrophages stimulated B1 cells to produce IgM antibodies to a similar extent. Thus, the increase of IgM in LDLR$^{-/-}$ mice on a HFD is not due to a different stimulation capacity by macrophages (**Fig. 15-b**).

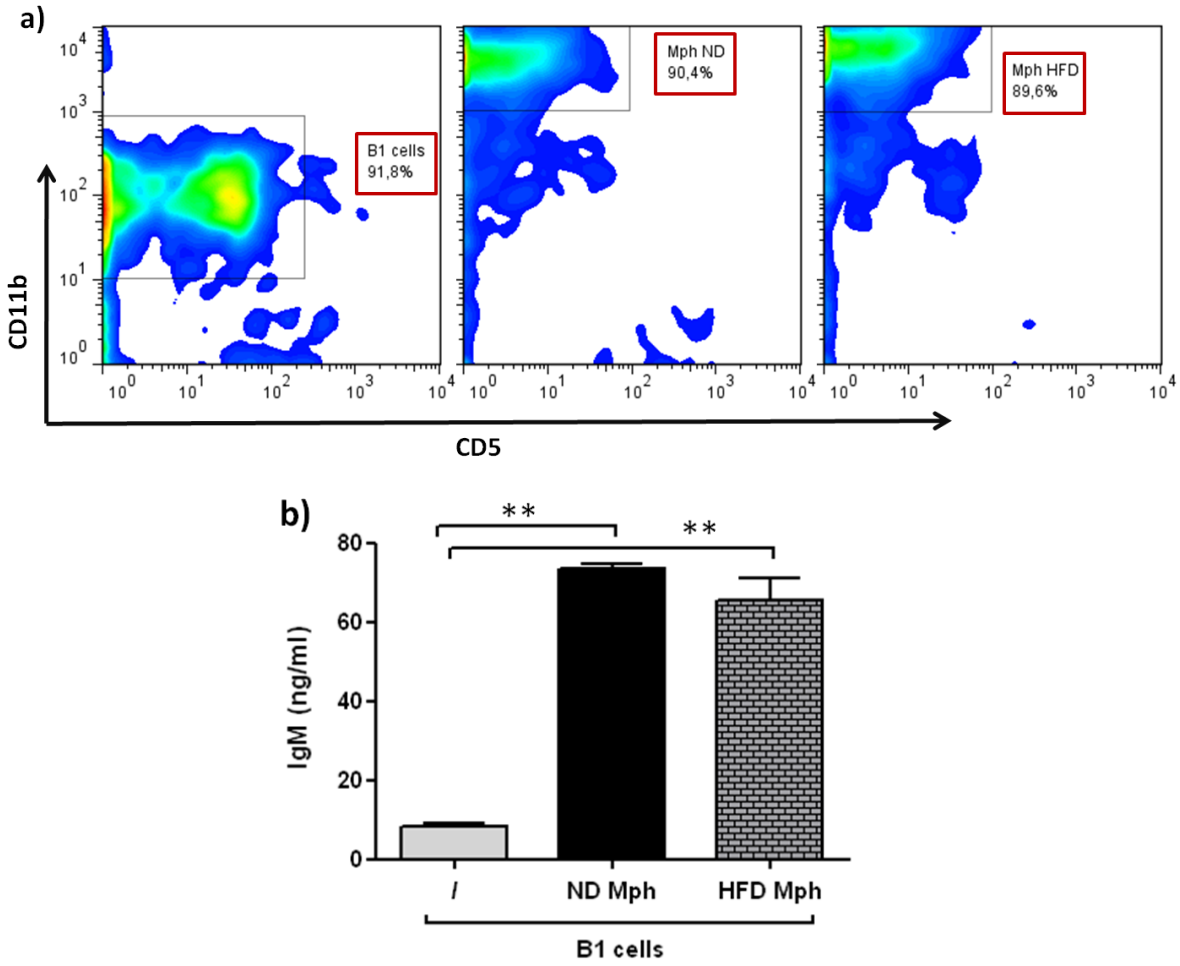


Fig. 15: IgM secretion in B1 cell co-culture with macrophages from mice on HFD and ND. a) Flow cytometry plots for the purity of sorted cell populations. The purity of the B1 cells obtained from a C57BL/6 mouse was 93%. Macrophages from mice on HFD had a purity of 90% and those from mice on ND were 91% pure. **b)** Comparing the IgM secretion stimulation capacity of macrophages obtained from $LDLR^{-/-}$ mice on ND and HFD. After 72h both ND (73,5ng/ml) and HFD (65,6ng/ml) macrophages induce IgM secretion of B1 cells compared to B1 cells (8,5ng/ml) cultured alone. There was no significant difference between the macrophages on different diets. Data are presented as mean ± SEM of 3 replicates, ** indicates p<0,005.

aBAFFR treatment of B1 cells in presence of macrophage and T cells

Because the effect of the BAFFR blocking on IgM production by sorted B1 cells was less robust compared to the effect when total peritoneal cells were used, I wanted to examine whether aBAFFR treatment affects IgM secretion in the presence of other cell types. B1 cells, T cells and macrophages were isolated from the peritoneal cavities of C57BL/6 mice by FACSorting (purity of the populations **Fig. 10-a**), and cultured in different combinations with other cells. In addition the effect of the aBAFFR antibody on IgM secretion under these specific experimental conditions was evaluated.

Unfortunately, I only succeeded once in performing this experiment and additional repeat experiments would be needed. However, the preliminary results suggest that in the presence of macrophages IgM secretion of B1 cells is up regulated, but when T cells are added to this co-culture the increased IgM production is reduced. T cells alone have no effect on IgM secretion by B1 cells. In presence of aBAFFR antibody, the increased production of IgM antibodies induced by macrophages is reduced. In contrast, negative effect of T cells in this culture system is reversed by aBAFFR antibody. Thus, this suggests that blockage of the BAFFR decreases the capacity of macrophages to stimulate IgM secretion. T cells seem to suppress IgM production of B1 cells in presence of macrophages and addition of aBAFFR removes this suppression. (**Fig. 16**)

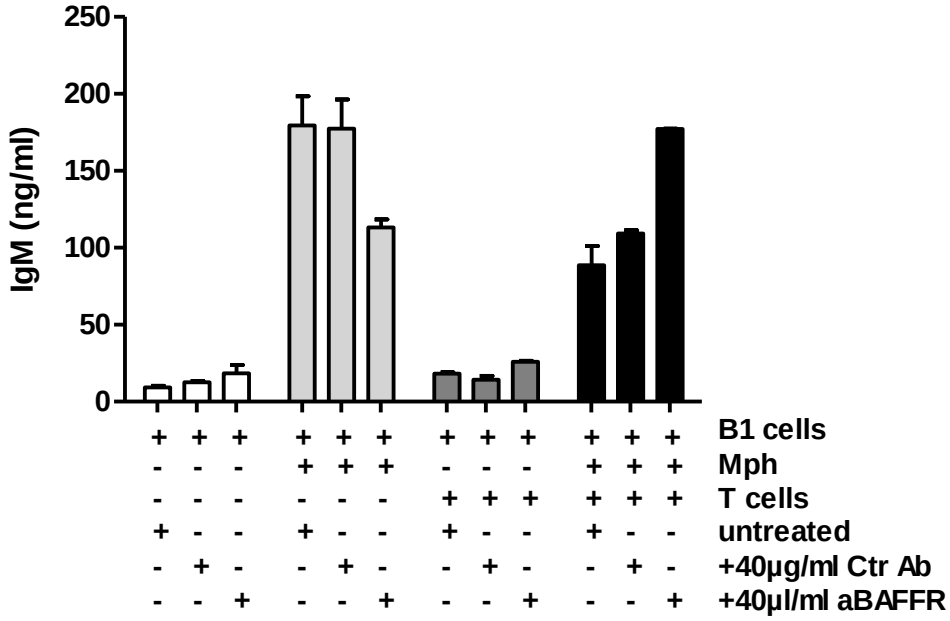


Fig. 16: aBAFFR treatment of co-culture of B1 cells with macrophages and T cells. Macrophages increase IgM secretion but in presence of aBAFFR the titers decrease. When macrophages and T cells are present the titers are decreased compared to only presence of macrophages, but when aBAFFR is added the titers increase again to the levels achieved when only B1 cells and macrophages are present.

DISCUSSION

The dependence of B2 cell survival on the BAFF-BAFFR interaction is very well documented, while its role for B1 cells has not been described, except for the fact that it is not a determinant for B1 cell survival. As part of a mouse study performed in the laboratory, in which the role of BAFF neutralization in atherosclerosis is examined, the role of BAFF signaling on B1 cells functions needed to be studied and therefore was the focus of my diploma thesis.

During the performance of the practical part I discovered two hitherto unrecognized pathways concerning IgM secretion by B1 cells. The first finding revealed the role of BAFF-BAFFR signaling in peritoneal B cells on IgM secretion in vitro. In many independent assays I could show that neutralization of this signaling pathway with an anti-BAFFR blocking antibody resulted in increased IgM production. I confirmed specific binding of the antibody and also excluded any endotoxin side effects that could have acted on the IgM secretion as well.

In attempts to understand how IgM secretion is promoted by blockage of the BAFFR, I first evaluated the possibility that an interaction of BAFF ligand with its other receptor TACI was involved. In such a scenario blockage of the BAFFR would allow more BAFF to bind TACI and this interaction may stimulate IgM production. However, my data do not support this, as adding excess recombinant BAFF protein to the cultures in vitro did not alter the IgM production.

Importantly, I also could confirm this very interesting and novel effect of enhanced IgM production using an anti-BAFF antibody (GSK), which further verified that the observed effect is not dependent on TACI, as the ligand is neutralized by the antibody and therefore not able to interact.

I could not unmask increased expression of genes associated with IgM secretion, such as XBP-1 and Blimp-1, in response to BAFFR neutralization. In addition, I compared serum BAFF titers in $LDLR^{-/-}$ mice on HFD to those on ND, as they have increased serum IgM

titers. However, I did not observe a difference between mice on ND and HFD and therefore the increase in IgM in this case is unlikely linked to BAFF-BAFFR signaling.

BAFFR blocking on B1 cells alone in culture slightly stimulates their IgM secretion but not to the extend seen in total peritoneal cell cultures. This raised the question if other cells are involved in the observed effect. Interestingly, my second discovery identified the fact that macrophages promote IgM secretion by B1 cells, independently of aBAFFR antibody treatment. Using primary sorted cells as well as in cell lines, I could further show that this stimulation in vitro is dependent on direct cell contact. This raised the question whether the spontaneous secretion of IgM in physiological conditions is maintained through macrophages and whether IgM secretion would be lower in their absence. Taking this into account I assumed there might be a difference in the stimulation capacity of macrophages coming from $LDLR^{-/-}$ mice on ND and HFD, as dramatically increased IgM titers are found in mice on a HFD. However, I could not identify differences in IgM antibody secretion by peritoneal B1 cells stimulated with peritoneal macrophages obtained from HFD or ND mice, respectively.

With respect to the increased IgM secretion induced by aBAFFR antibody in total peritoneal cells, I could show that BAFFR blocking decreased IgM production in co-cultures where both B1 cells and macrophages were present. When B1 cells, macrophages and T cells were present the titers were also lower than those when only B1 and macrophages were present. However, when the BAFFR was blocked in presence of all three cell types the titers increased. This suggests that macrophages have the ability to stimulate IgM secretion and that this effect is down-regulated when the BAFFR is blocked. In the presence of T cells the stimulatory capacity of macrophages is down-regulated, but once the BAFFR is blocked this effect is reversed.

Another very exciting finding I obtained is the potential expression of the BAFFR on macrophages. So far BAFFR expression on B cells has been described, but no indication for expression on macrophages exists in the literature. I could demonstrate expression of the BAFFR on primary peritoneal macrophages as well as RAW macrophages. Future studies will need to evaluate the functional role of BAFFR signaling in macrophages.

BAFF-BAFFR signaling could activate macrophages to secrete stimuli or express surface molecules that have the capacity to interact with B cells. In addition, there is evidence

that endothelial cells express BAFF therefore one could hypothesize that monocytes/macrophages can interact with endothelial cells in a BAFF-BAFFR dependent manner.

The finding described above indicate a beneficial role of BAFF-BAFFR neutralization in atherosclerosis as it could lead to the depletion of proatherogenic B2 cells but also an increased secretion of atheroprotective IgM by B1 cells thereby resulting in less atherogenesis.

IgM Enzyme-linked immunosorbent assay (ELISA)

Buffers and Solutions:

- *PBS (pH 7.4, 1l):*

  1,31g NaH2PO4

  5,75g NA2HPO4

  8,765g NaCl

- *PBS-Tween (1l):*

  1l PBS

  1ml Tween20 (50%)

- *10xTBS (pH 7.4):*

  0,15M NaCl

  50mM Tris

  0,27mM EDTA

  0,02% Azide

- *Dilution and Blocking Buffer:*

  TBS

  1%BSA (RIA grade, SIGMA)

- *Lumiphos (Lumigen, Inc.)*

Antibodies:

- Coating antibody: anti-mouse-IgM (µ-chain specific), developed in goat, affinity isolated antigen specific antibody, (SIGMA)
- Isotype Control: mouse IgM, κ, concentration 0.5 mg/ml , (BIOLEGEND)
- Secondary Antibody: anti-mouse-IgM (µ-chain specific)-Alkaline phosphatase, developed in goat, (ZYMAD)

Procedure:

96-well U-bottom Elisa plates were coated with 40µl/well coating anti-mouse IgM (µ-chain specific) antibody, diluted to 5µg/ml in 1xPBS, and incubated over night at 4°C (or 1h at RT). Plates were washed 3x with PBS (BIO-TEK plate washer) and wells blocked with 80µl/well BSA/TBS for at least 30min at RT. After washing plates with PBS-Tween, 30µl of samples and standards (isotype control: from 50 - 0,7812ng/ml) diluted in BSA/TBS were applied always in technical triplicates and incubated for 2h at RT (or o/n at 4°C). Plates were washed 3x with PBS-Tween and 40µl of secondary antibody, diluted 1:20 000 in BSA/TBS was added to each well for a 1h incubation at RT. Thereafter, plates were washed 3x with TBS, and 25µl of Lumiphos (diluted 1:3 in H_20) per well were applied for 60min at RT in darkness. Relative light units were measured with a Biotek Synergy 2 ELISA reader and analyzed with Microsoft Office Excel 2007, and significance was tested with GraphPadPrism 5.04 (GraphPad Software).

BAFF-ELISA:

For BAFF titer measurements in the sera of mice the Quantikine mouse BAFF/BLyS/TNFSF13B Immunoassay (R&D Systems) was used. $LDLR^{-/-}$ mouse serum samples were diluted 1:6 in the buffer provided in the kit and measured in technical duplicates. In total the titers were measured in sera of 11 mice.

Western Blot:

Buffers and Solutions:

- *1xTBS-T:*

  100ml 10xTBS

  2ml Tween20

- *10x TBS:*

  12,1g Tris

  87,6g NaCl

  pH 7,5

- *4x Lower Buffer:*

  1,5M Tris Hcl

  0,4% SDS

  pH 8,8 (HCl)

- *4x Upper Buffer:*

  0,5M Tris HCl

  0,4% SDS

  pH 6,8

- *10x Running Buffer (1L):*

  144g Glycin

  30g Tris

  10g SDS

  dH2O (pH 8,3)

- *10x Transfer Buffer:*

  250mM Tris

  1,92M Glycine

- *1xTransfer Buffer (1L):*

  100ml 10xTransfer Buffer

  100ml MeOH

  dH20

- *1xTBS-T:*

  100ml 10xTBS

  2ml Tween20

- *Lower Gel 1,5mm:*

  3ml 30% Acrylamide

  4,5ml H2O

  2,5ml 4x Lower Buffer

  100µl Ammonium *Persulfate*

  8µl TEMED

- *Upper Gel 1,5 mm:*

  0,6ml 30% Acrylamide

  0,8ml 4x Upper Buffer

  1,8ml dH2O

  32µl Ammonium Persulfate

  2,5µl TEMED

- 2x Laemli Lysis Buffer:

  4% SDS

  20% glycerol

  10% 2-mercaptoethanol

  0,004% bromphenol blue

  0,125M Tris HCL

- ECL Plus Western Blotting Detection System (GE Healthcare)

Sample preparation:

Splenocytes from C57Bl/6 mice and BCL-1 cells were lysed in Laemmli lysis buffer, heated at 100°C and vortexed well before loading on the gel.

Procedure:

The lower part of the gel was poured in gel cassette and covered with 1ml Isopropanol, which was removed by washing with H_2O after gel polymerization. The upper part of the gel was poured on top of the lower gel and mounted with a slot comb, which was removed after polymerization. The cassette was placed in an electrode chamber filled with Running Buffer. 10µl of cell lysates were loaded on the gel. Proteins were separated in a run at 100V (~1h). After removal of the upper gel the lower part of the gel was transferred on a nitrocellulose membrane. The transfer was performed in a Bio-Rad Trans-Blot Semi-Dry System at 15V for 30min. Membranes were blocked with TBS-T/3%BSA o/n at 4°C (or for 2h at RT). First, target specific antibodies and secondary detection antibodies were diluted in TBS-T/3%BSA, applied for 1h one after each other and in between membranes were washed 3 times with TBS-T for 10min. 1ml of ECL Plus solution was administered to the membranes for 5min before exposure. Pictures were taken and analyzed with ImageLab (BIO-RAD).

FACS:

Buffers and Solutions:

- *FACS- Buffer:*

  PBS

  2% FBS

Staining antibodies:

- aCD16/32 CD16/32 (FCGR3, IGFR3, FCGR2, FC Receptor Block) Rat IgG2a, λ, mouse CD16/CD32, (used 1:200), (eBioscience)
- aCD5 PE, Rat IgG2a, κ, mouse CD5, (used 1:100), (eBioscience)

- aCD11b APC Rat IgG2b, κ, mouse CD11b, (used 1:800), (eBioscience)
- aCD45R(B220) PerCP-Cy5.5, Rat IgG2a, κ, CD45, (used 1:800), (eBioscience)
- CD268 (BAFF Receptor) FITC, Rat IgG1, κ, mouse CD268, (used 1:800), (eBioscience)

All antibodies were diluted in FACS-Buffer.

Cell Sorting:

Total peritoneal cells were isolated from C57BL/6 (or $LDLR^{-/-}$) mice by peritoneal lavage (see animal handling) and blocked with 800µl mouse anti-CD16/32 for 20min on ice. After washing 3x with FACS-Buffer, cells were resuspended in 800µl antibody mix (CD5-PE, B220-PerCP Cy5.5 and CD11b-APC) and incubated for further 20min at 4°C in darkness. Additionally, for each cell surface marker single stainings with antibodies labeled with a distinct fluorescent dye were prepared for laser compensation. Afterwards cells were washed 3x with FACS-buffer and sorted with FACSAria (BD Biosciences) according to their cell surface marker expression. Purities of the populations were confirmed with FACSCalibur and FlowJo- Flow Cytometry Analysis Software (TreeStar). Subsequently, cells were used for BAFFR staining or cell culture experiments (see below).

B1: B1a - $B220^{+}$, $CD11b^{+}$, $CD5^{+}$ and B1b - $B220^{+}$, $CD11b^{+}$, $CD5^{-}$;
Macrophages: $B220^{-}$, $CD11b^{high}$, $CD5^{-}$;
T cells: $B220^{-}$, $CD11b^{-}$, $CD5^{+}$;

Staining for BAFFR expression on peritoneal cells:

-Unsorted PEC: 500,000 isolated cells were blocked with 100µl aCD16/32 antibody for 20min on ice and after washing 3x with FACS-Buffer stained in 100µl antibody mix (BAFFR-FITC, CD5-PE, B220-PerCP Cy5.5 and CD11b-APC) for 20min on ice. Finally, cells were washed 3x with FACS-buffer.

-Sorted PEC: approximately 50,000 B1 cells, macrophages and T cells obtained from cell sorting were incubated in 100µl aBAFFR-FITC antibody for 20min on ice and washed 3x with FACS-Buffer.

Flow cytometry was performed using a FACSCalibur machine (BD Biosciences) and data were analyzed using FlowJo- Flow Cytometry Analysis Software (TreeStar).

Staining for BAFFR expression on RAW and BCL-1 cell line:

Approximately 500,000 cells of each cell line were blocked with 100µl anti-CD16/32 antibody (diluted 1:200) for 20min on ice. After washing 3x with FACS-buffer and pelleting for 5min at 1300 rpm, cells were resuspended in 100µl anti-BAFFR-FITC antibody (diluted 1:800), incubated for 20min on ice, and washed 3x with FACS-buffer. Stained cells were analyzed on a FACSCalibur (BD Biosciences) instrument and data processed using FlowJo- Flow Cytometry Analysis Software (TreeStar).

<u>In vitro experiments:</u>

***Media*:**

- Dulbecco's Modified Eagle's Medium - DMEM (GIBCO)
- RPMI - Roswell Park Memorial Institute (GIBCO)
- Dulbecco's Phosphate-Buffered Saline D-PBS (SIGMA)

Cell lines:

- *Mouse leukemic monocyte macrophage cell line - RAW 264.7:*

A mouse cell line derived from the ascites of a tumor induced in male BALB/c mice by intraperitoneal injection of Abselon Leukaemia Virus (A-MuLV). Cells are typically adherent and mainly used in metabolic, inflammation and apoptosis studies. RAW 264.7 were cultured at 37°C in DMEM media containing 10% heat inactivated Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin, and 1% Glucose. For maintenance, cells were detached using a cell scraper (Fisher), pelleted for 5min at 1300rpm, and re-plated 1:4 in a fresh culture dish.

- *B cell Lymphoma 1 - BCL-1 :*

A mouse cell line established from a spontaneous lymphoma obtained from a female BALB/c mouse. This cell line is most likely of B1a origin and expresses CD5, surface IgM,

Mac-1, CD43 and low level of B220. Bcl-1 cells were cultured at 37°C in RPMI media containing 10%FBS, 1% Penicillin/Streptomycin, 1% Sodium Pyruvate, and 0,1% beta-mercaptoethanol. Cells adhere lightly but can be detached easily by flushing the flask with media.

Cell Counting:

Cells were counted using a CASY® Technology Cell Counter from Roche Innovatis AG.

Antibodies:

- Anti-mouse BAFFR 9B9-Hybridoma antibody (by Rolink (102); antibody purification by BIOTEM)
- Isotype matched control antibody: rat IgG2b
- Recombinant mouse BAFF protein (R&D Systems)
- Hamster Anti-mouse-BLyS/BAFF (GSK, HGS)
- Anti-mouse CD40 (eBioscience)

aBAFFR treatment of total peritoneal cells:

Total peritoneal cells from a C57Bl/6 mouse were isolated, and 100,000 cells per well were placed in a 96-well plate in 200µl serum free DMEM media. Secretion of untreated cells was compared to that of cells treated with 20µg/ml and 40µg/ml of anti-BAFFR or control antibody. Each experimental condition was performed in biological triplicates, and cells were incubated for 48h, 72h or 96h at 37°C / 5% CO_2. Cells were removed by 5min centrifugation at 1,300 rpm and supernatants were used for IgM titer measurement by ELISA.

Exclusion of endotoxin side effects:

50,000 peritoneal cells from C57BL/6 mice per well were plated in serum free DMEM media in a 96-well plate. PMB was added in different concentrations to the antibodies (10µg/ml, 20µg/ml, 30µg/ml) and incubated for 1h at 37°C. 20µg/ml and 40µg/ml of anti-BAFFR or control antibody were added to the cells, and cultured at a final volume of 200µl per well for 48h at 37°C / 5% CO_2. Supernatants were assayed for IgM.

Anti-BAFF treatment of total peritoneal cells:

PEC were isolated from C57BL/6 mice, and 50,000 cells in 200µl serum free DMEM media per well were plated in 96-well cell culture plates. Three different conditions in biological quadruplicates were compared: untreated cells, cells treated with 40µg/ml anti-BAFFR, and cells treated with 10µg/ml anti-BAFF. Cells were incubated for 72h at 37°C / 5% $CO_2$and then pelleted for 5min at 1,300 rpm to obtain cell free supernatant for further analyses.

Anti-BAFFR treatment proliferation assay:

PEC were isolated from C57BL/6 mice and 100,000 cells in 200µl serum free DMEM per well were plated in 96-well culture plates. Cells were treated with 40µg/ml aBAFFR, 40µg/ml control antibody, or left untreated and incubated at 37°C / 5% CO_2. After 48h the cells were counted, stained will specific antibodies for flow cytometry, and percentages of different cell populations of total living cells were quantified.

Anti-BAFFR and BAFF treatment of sorted B1 cells:

B1 cells were obtained by sorting of peritoneal cells of C57BL/6 mice. 50,000 B1 cells were plated in 96-well plates in 200µl per well serum free DMEM media. 20µg/ml and 40µg/ml of anti-BAFFR or control antibody or 10ng/ml BAFF recombinant protein were added to the wells in triplicate cultures. In addition, cells were incubated with 40µg/ml anti-BAFFR and then 10ng/ml BAFF recombinant protein was added. Following 72h incubation at 37°C / 5% CO_2 IgM titers were measured in the supernatants.

Anti-BAFFR treatment of sorted peritoneal cells in co-culture:

Cells obtained from cell sorting were co-cultured in different combinations. B1 cells alone, B1 cells and macrophages, B1 cells and T cells and B1, macrophages and T cells together. 50,000 cells of each cell type was present per well in 200µl serum free DMEM media. Each combination was either untreated or treated with 20µg/ml or 40µg/ml anti-BAFFR or control antibody. Cells were incubated for 6 days at 37°C / 5% $CO_2$and the IgM titers in the supernatants were measured by IgM ELISA.

Titration of BCL-1 and RAW cells:

Different numbers of BCL-1 cells and RAW macrophages were co-cultured, starting from 0 to 150,000 cells per well from each cell line at different combinations. Cells were cultured in 200µl serum free DMEM media for 10h at 37°C / 5% CO_2. IgM was assayed by ELISA in cell supernatants.

Transwell Experiment for sorted peritoneal cells:

For the transwell experiments, HTS-Transwell 96-well plates with a 5.0µm Polycarbonate Membrane (CORNING) were used. 50,000 B1 cells were plated in the lower well and 50,000 macrophages were added in the second well on top. Results were compared to B1 cells cultured alone and B1 cells cultured in a 96-well cell culture plate together with 50,000 and 25,000 macrophages in a total volume of 200µl serum free DMEM media. The cells were incubated for 72h at 37°C / 5% CO_2 and IgM titers were measured in supernatants.

Transwell experiment for BCL-1 and RAW cells:

HTS-Transwell 96-well plates with a 5.0µm Polycarbonate Membrane (CORNING) were used. 50,000 BCL-1 cells were placed in the lower well and 50,000 RAW macrophages were added in the second well on top in a total of 200µl serum free DMEM media. Cells were incubated for 18h at 37°C / 5% CO_2 and the IgM titers were measured in the supernatants.

Comparing macrophage from ND vs. HFD LDLR$^{-/-}$ in terms of IgM stimulation:

B1 cells were obtained by cell sorting of peritoneal cells obtained from C57Bl/6 mice, and peritoneal macrophages were obtained from LDLR$^{-/-}$ mice that were either fed HFD for 12 weeks or remained on a ND. 50,000 of each cell type per well were plated in 200µl DMEM media in 96-well cell culture plates. Three different conditions in biological triplicates were compared: B1 cells alone, B1 cells cultured with macrophages from ND mice, and B1 cells cultured with macrophages from HFD mice. Cells were incubated for 72h at 37°C / 5% CO_2 and the supernatants were used for determination of IgM by ELISA.

Mouse handling:

Buffers and Solutions:

- *Hank's Buffered Salt Solution* – HBSS
- *Erythrocyte Lysis Buffer (Hämolysebuffer - Morphisto Evolutionsforschungund Anwendung GmbH)*

Mouse strains:

- *C57BL/6 mice*:

This mouse strain is an inbred strain that is most often used in the laboratory, as it is very robust and easy to bread.

- *LDLR$^{-/-}$ mice* (low density lipoprotein- receptor knock-out mice) on a C57BL/6 background:

This mouse strain is a generally accepted animal model for the examination of hyperlipidemia and atherosclerosis. By feeding mice a cholesterol-rich diet, cholesterol levels are increased in the serum due to the lack of LDL receptors, and consequently the formation of atherosclerotic plaques is promoted. Therefore, this model is appropriate to follow the progression/regression of atherosclerotic plaques.

Peritoneal Lavage:

C57BL/6 mice were sacrificed by cervical dislocation. After sterilization with 70% Ethanol a small incision in the abdominal skin of the mouse was made using one set of forceps and scissors to access the peritoneal cavity. Per mouse 10ml of *HBSS* was injected in the lower abdominal region, to avoid organ puncture, using sterile 10ml syringes. Peritoneal cells were obtained by lavage and aspirated using a fresh 10ml syrine. Cells were recovered by 5min centrifugation at 1,300 rpm.

Splenocyte isolation:

The spleens of C57BL/6 mice were isolated with a pair of forceps and scissors and single cell suspensions were made by passing the tissue through a 100µm nylon cell strainer (Fisher Scientific). Erythrocytes were lysed by adding 1ml of red blood cell lysis buffer for

2min incubation at RT. To stop the reaction 10ml of ice cold PBS were added and cells pelleted for 5min at 1,300 rpm.

RNA-Extraction:

Peritoneal cells from the anti-BAFFR treatment experiment were lysed in RNA-Lysis Buffer and then RNA was isolated using the peqGOLD total RNA isolation kit (PEQLAB). The obtained RNA was diluted 1:10 in RNase free water before it was used to generate cDNA .

Preparation of cDNA:

cDNA was synthesized using high capacity cDNA RT kit (Applied Biosystems). First a master-mix was prepared, containing buffer, dNTPs, Oligos and H_20. Afterwards RNA obtained from the RNA isolation was added and Reverse Transcriptase was applied.

- *1x Reverse Transcription*

  1µl Buffer

  0,4µl dNTPs

  1µl Oligos

  0,5µl Reverse Transcriptase

  2,1µl H20

  5µl RNA

Program:

Cycles:
1. 25°C for 25min
2. 37°C for 120min
3. 85°C for 5sec
4. 4°C unlimited

RT-PCR:

For the identification of expression levels of XBP-1 and Blimp-1, IQ Syper-Green Supermix (BIORAD) was used. The PCR was carried out in the BIORAD Thermal Cycler C1000 (CFX96 Real Time System).

- *1x PCR:*

  5µl Buffer (Syber Green)

  0,5µl Primermix (Forward: Reverse = 1:1)

  4,5µl cDNA (1:10)

Sequences and primers:

- XBP-1: (5′-A G C A C T C A G A C T A T G T G C A C C TCT -3′, 5′-T C C A G A A T G C C C A A A A G G A T A T C -3′)
- Blimp-1: (5′-A G T A G T T G A A T GGG A G C -3′, 5′-C A A T G C T T G T C T A G T G T C -3′)

Program:

- 1 cycle 50°C (2:00min);
- 1 cycle 94°C (10:00min);
- 40 cycles 95°C (0:15min) – 60°C (0:15min) – 72°C (0:45min)
- 1 cycle 95°C (0:15min) – 60°C (0:15min) – 95°C (0:15min)

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POSTERS:

Natural IgM antibodies suppress the pro-inflammatory effect of apoptotic blebs in atherosclerosis

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