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The influence of IL-38 in the process of B cell differentiation

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AID	Activation-induced cytidine deaminase
AP-1	Activator protein 1
AS	Ankylosing spondylitis
BAFF	B cell activating factor
BCL6	B cell lymphoma 6
BCR	B cell receptor
BMDCs	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
CD	Crohn´s disease
CD40L	CD40 ligand
CRP	C-reactive protein
FCS	Foetal calf serum
FDC	Follicular dendritic cells
FLS	Fibroblast like synoviocytes
IBD	Intestinal bowel diseases
ICOS	Inducible T cell co-stimulator
IFN-α/γ	Interferon α/γ
Ig	Immunoglobulin
IgH/IgL	Immunoglobulin heavy chain/light chain
IL-1F	Interleukin-1 family of cytokines
IL-1R	IL-1 Receptor
IL-1RAPL1	IL-1 Receptor Accessory Protein Like 1
IL-36R	IL-36 Receptor
IRF4	Interferon regulatory factor 4
JNK	c-Jun N-terminal kinases
LPS	Lipopolysaccharides
mABs	mouse Antibodies
MAPK	Mitogen associated protein kinase
MBC	Memory B cell
MMP-2/9	Matrix metalloproteinase-2/-9

mnBC	Mature naïve B cell
NFκB	Nuclear factor kappa B
NLRP3	NLR family pyrin domain containing 3
NLS	Nuclear localization sequence
PAX5	Paired box protein 5
PB	Plasmablast
PC	Plasma cell
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PRDM1	positive regulatory domain containing 1
rhIL-38	recombinant human IL-38
SEM	Standard error of the mean
SLE	Systemic Lupus Erythematosus
STAT3	Signal transducer and activator of transcription 3
TIR	Toll/interleukin-1 receptor
TNF-α	Tumour necrosis factor α
TLR	Toll-like receptor
UC	Ulcerative colitis
XBP1	X-box-binding protein 1

1. Summary

IL-38 is the latest discovered cytokine of the IL-1 family and has been added to the IL-36 subfamily. Since its discovery in 2001, increasing evidence suggests predominantly anti-inflammatory properties of IL-38, which are most likely exerted through three potential receptors, the IL-1 Receptor 1 (IL-1R1), IL-36 Receptor (IL-36R) and the IL-1 Receptor Accessory Protein Like 1 (IL-1RAPL1). However, to this date detailed knowledge of IL-38 functioning remains to be examined. Importantly, how IL-38 is processed, secreted from cells and the exact mechanisms of target receptor binding and intracellular signaling are not fully understood. Further, IL-38 has been associated with regulatory functions in autoimmune diseases like systemic lupus erythematosus (SLE) and psoriasis. At the same time however, connections between B cells as indispensable part of immunity and IL-38 remain rare.

In this study we examined the influence of IL-38 in peripheral human blood B cells differentiating into antibody secreting cells using a three-step in vitro differentiation process. We first show that all potential IL-38 binding receptors are present on peripheral blood B cells on a gene expression level and remain detectable throughout B cell differentiation. Next, while B cells treated with exogenous IL-38 depict no differences in early B cell activation markers, the process of B cell differentiation revealed significant alterations in B cell phenotype created by IL-38 treatment. Predominantly on day 7 of the differentiation process, IL-38 treated B cells showed significantly reduced CD38 expression which depicts an important step in development towards plasma cells. We hypothesize that IL-38 acts antagonistically on the IL-1R1 pathway reducing Nuclear factor kappa B (NFκB) expression and consequently decreasing CD38 expression. Further IL-38 reduced early antibody production while increasing IgM secretion at the end stages of differentiation. Next, we repeated the differentiation assays under the influence of additional IL-21 stimulation to further enhance plasma cell development. In these experiments, the impact of IL-38 on B cell differentiation and immunoglobulin production were reduced, indicating a comparatively moderate relevance of IL-38 for B cell differentiation. We then examined how proliferation and cell death were impacted by exogenous IL-38 during B cell

differentiation. IL-38 treatment alone significantly reduced B cell survival which was further augmented by IL-21 stimulation. We conclude that IL-38 and IL-21 act synergistically in promoting B cell apoptosis, also depicting an anti-inflammatory property of IL-38. Finally, using a siRNA we successfully performed an IL-38 knockdown experiment of human blood B cells reducing IL-38 expression to 44% measured on day 4 of B cell differentiation. In these experiments we observed reversed tendencies of CD38 expression compared to exogenous IL-38 treatment. Here, IL-38 knockdown cells showed increased CD38 expression indicating endogenous regulatory properties of IL-38 in B cell differentiation.

Our project, for the first time proves direct effects of IL-38 on human B cells. The results support previous research of IL-38 to act anti-inflammatory as it seems to modulate B cell differentiation, survival, and immunoglobulin production in a down-regulatory manner. These findings pave way for more detailed research on the connection between B cell homoeostasis and IL-38 function.

2. Zusammenfassung

IL-38 ist das zuletzt entdeckte Zytokin der IL-1 Familie und wurde der Subgruppe der IL-36 Zytokine zugeordnet. Seit der Entdeckung von IL-38 im Jahr 2001 ergeben sich zunehmend Hinweise einer vorwiegend anti-inflammatorischen Wirkung des Zytokins, die am ehesten über drei Rezeptoren – IL-1 Rezeptor 1 (IL1-R1), IL-36 Rezeptor (IL-36R) und IL-1 Receptor Accessory Protein Like 1 (IL-1RAPL1) – entfaltet wird. Die genaue Wirkweise des IL-38 ist bis heute jedoch noch unklar. Insbesondere die intrazelluläre Prozessierung, Sekretion, Rezeptorbindung und weiterführende Signalkaskaden sind noch nicht entschlüsselt worden. Nichtsdestotrotz scheint IL-38 eine regulierende Bedeutung im Rahmen verschiedener Autoimmunerkrankungen wie dem Systemischen Lupus Erythematosus (SLE) oder der Rheumatoiden Arthritis (RA) zu haben. Gleichzeitig ist die Beziehung von IL-38 und B Zellen als signifikanter Bestandteil des Immunsystems noch unklar.

In der vorliegenden Arbeit untersuchten wir den Einfluss von IL-38 im Prozess der Differenzierung peripherer humaner B Zellen des Blutes zu Antikörper produzierenden Zellen mittels drei-schrittigem in vitro Protokoll. Hierbei zeigen wir zunächst, dass alle potenziell IL-38 bindenden Rezeptoren auf B Zellen des peripheren Blutes auf Genexpressionsebene nachweisbar sind. Anschließend zeigen wir, dass B Zellen, die mit exogenem IL-38 behandelt werden zwar keine phänotypischen Unterschiede bezüglich klassischer B Zell Aktivierungsproteine aufweisen, der Prozess der B Zell Differenzierung jedoch verändert wird. Hauptsächlich zeigt sich dies an Tag 7 der Differenzierung in Form einer signifikant reduzierten CD38-Exprimierung der mit IL-38 behandelten Zellen, die einen wichtigen Schritt in der Differenzierung in Richtung Plasmazellen darstellt. Wir stellen die Hypothese auf, dass IL-38 antagonistisch über den IL-1R1 wirkt und durch eine Reduktion des Nuclear factor kappa B (NFκB) die Expression des CD38 Oberflächenproteins mindert. Darüber hinaus zeigt sich eine IL-38 bedingte Reduktion der frühen Antikörperproduktion bei jedoch erhöhter IgM-Sekretion am Ende der B Zell-Differenzierung. Als nächstes wiederholten wir die B Zell-Differenzierungsversuche unter zusätzlicher IL-21-Stimulation, um eine erhöhte Zahl an Plasmazellen zu generieren. Hierbei zeigte sich eine deutliche

Verringerung der zuvor gesehenen Effekte von IL-38 auf die B Zell Subgruppen und Immunglobulin Produktion, sodass die biologische Relevanz IL-38 bezüglich der B Zell-Differenzierung im Vergleich zu anderen Zytokinen geringer einzuschätzen erscheint.

Des Weiteren untersuchten wir, wie die B Zell-Proliferation sowie Zelltod von exogenem IL-38 beeinflusst werden. Hierbei war die alleinige IL-38-Stimulation mit einem verringertem B Zell Überleben verbunden, was durch zusätzliche IL-21-Stimulation noch verstärkt wurde. Wir folgern, dass IL-38 synergistisch mit IL-21 die Apoptose von B Zellen fördert und damit ein anti-inflammatorisches Signal darstellt.

Als letztes gelang es uns mittels siRNA einen erfolgreichen IL-38 Gen-Knockdown zu kreieren, der das IL-38 Expressionsniveau an Tag 4 der Zell-Differenzierung auf 44% absenkte. In diesen Experimenten zeichnete sich die Tendenz einer erhöhten CD38-Exprimierung in den Zellen mit verminderter IL-38-Expression ab und somit im Gegensatz zu den vorherigen Beobachtungen der exogenen IL-38 Wirkung steht. Insgesamt erscheint daher, neben der exogenen Funktion des IL-38 auch eine relevante endogene Regulationsfunktion vorzuliegen.

Die vorliegende Arbeit zeigt erstmals einen direkten Effekt des IL-38 auf humane B Zellen. Zudem geben unsere Ergebnisse, passend zu bisherigen Untersuchungen, weiteren Anhalt einer anti-inflammatorischen Funktion in Bezug auf Modulierung der B Zell-Differenzierung, des Zellüberlebens und der Immunglobulin-Produktion. Diese Ergebnisse ebnen den Weg für detailliertere Untersuchungen der Beziehung zwischen IL-38 und B Zell Funktionen.

3. Introduction

3.1 The biology of IL-38

3.1.1 IL-1 Family of cytokines

The family of interleukin-1 cytokines (IL-1F) depicts indispensable stimuli for the function of the immune system and during processes of inflammation.[1] Most IL-1F cytokines exert inflammatory effects, some are known to be anti-inflammatory. Today, eleven members of the IL-1F have been characterized and further categorized into three sub-families according to the length of their precursor molecules (**Figure 1**). There are ten receptors known to bind IL-1F cytokines.[2] In the cytoplasmatic domain of these receptors, the same functional domain was found which is also present in the Toll protein of Drosophilia.[3] This domain was termed Toll/interleukin-1 receptor (TIR) domain for having high resemblance with the one present in Toll-like receptors (TLR). Consequently, IL-1 receptors also have comparable functions as TLRs in initiating fundamental processes of inflammation and activating innate immune responses.[4] The latest addition to the IL-1F is IL-38. Its biological function remains to be understood in detail.

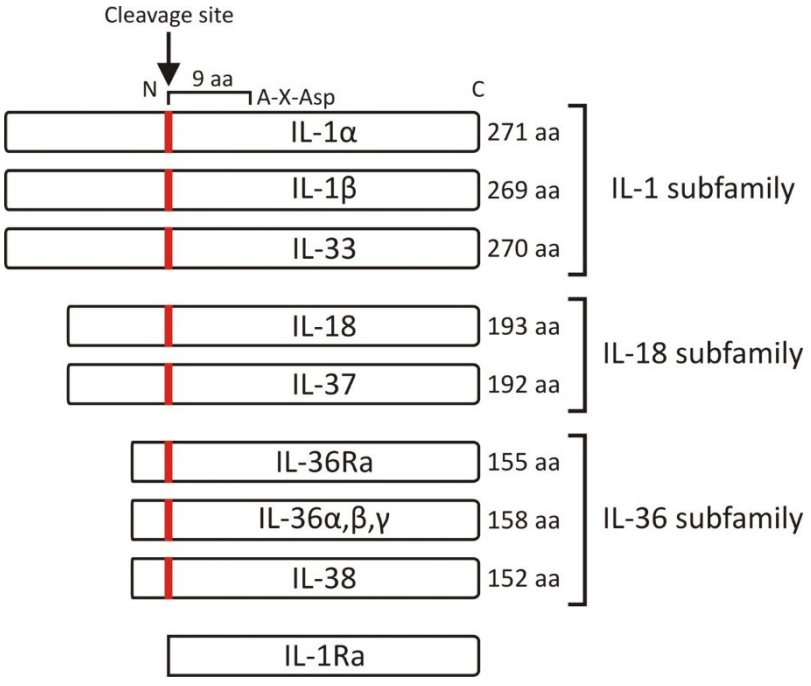


Figure 1: IL-1 sub-families
The IL-1F consists of 11 members, which have further been divided in three sub-families according to the length of their precursor. Except IL-1Ra, all IL-1F members contain a cleavage site to be N-terminally processed and further lack a signal peptide for protein secretion.

3.1.2 Biological characteristics of IL-38

In 2001, IL-38 was discovered and characterized as a new member of the IL-1 family. It was found located on chromosome 2q12-14 and originally named IL-1HY2 or IL-1F10. [5,6] The IL-38 gene is also part of the IL-1F gene-cluster, which contains all genes of the IL-1F members except IL-18 and IL-33.[7] It is especially closely located to the genes encoding IL-1Ra and IL-36Ra.[7] IL-38 gene produces a protein of 152 amino acids[6] and 16.9 kD molecular mass.[8] It has been observed in two forms, a full-length (aa 1-152) and few truncated (aa 3-152, 7-152, 20-152) versions.[9] The protein shares 43% homology with IL-36Ra, 41% with IL-1Ra,[6] and lower percentages with other IL-1F members, indicating a certain functional similarity with the aforementioned cytokines. IL-38 expression has particularly been described in skin epithelial cells but is also present in lymphatic tissues. Immunohistochemistry of human tonsils revealed IL-38 expression in proliferating B cells of the germinal center.[5] Recently, de Graaf et al depicted peripheral human blood B cells (PBMCs) as the main producer of circulating IL-38 among human PBMCs and that IL-38 plasma levels positively correlate with different B cell subsets like memory B cells and plasmablasts.[10] The B cell related function of IL-38 however remains unclear. Moreover, spleen, thymus, foetal liver, heart and placenta have been added to the tissues known to express IL-38.[6] So far it is not completely understood how endogenous IL-38 is intracellularly processed and how it is secreted. IL-38 is released from cells without containing a signal peptide[11] and does not have a nuclear localization sequence (NLS).[5] These findings are common for IL-1F proteins. Generally, IL-1F members and the IL-36 sub-family (IL-36α, IL-36β, IL-36γ, IL-36Ra) in particular, are processed to attain full biological activity.[12] They are cleaved 9 amino-acids before an A-x-Asp motif, being a highly conserved motif in the IL-1F.[12] IL-38 also contains this motif in its amino acid sequence, indicating it is also cleaved to modify its activity. Due to the length of its precursor domain IL-38 was categorized as member of the IL-36 sub-family.[13] Further, IL-38 does not contain a consensus cleavage site for Caspase-1, which is also consistent with the remaining IL-36 sub-family proteins.[14] Mora et al. found the truncated version of IL-38 to be released by human cancer cells and PBMCs under apoptotic cell culture conditions after being modified at its N-terminus.[9] Garraud et al. confirmed these results with apoptotic THP-1 cells, but also observed this under necrotic conditions. They also suggested different

proteases and cleavage sites for IL-38 processing, emphasizing matrix metalloproteinase 2 (MMP-2) as possible protease as it fits the A-x-Asp motif used to generate the truncated form of recombinant human IL-38 (rhIL-38).[15] Nonetheless, unlike the other IL-36 sub-family-members, the proteases responsible for cleaving IL-36Ra and IL-38 are currently unknown and the exact cleavage site for IL-38 remains to be confirmed.

3.1.3 Receptors and signaling of IL-38

As a result of its homology with IL-1Ra and IL-36Ra it was speculated that IL-38 might also function as an antagonist on the IL-1 and the IL-36 receptor. Lin et al. demonstrated recombinant IL-38 extracted from Chinese Hamster Ovary cells is able to bind soluble IL-1R1 with lower binding affinity compared to IL-1Ra and IL-1β.[5] A few years later Van de Verdoonk et al. showed that IL-38 does not bind to immobilized IL-1R1 but binds IL-36R with similar affinity as IL-36Ra.[16] Mora et al. confirmed Van de Verdoonks´ findings concerning the ability to bind IL-36R and added another receptor as possible target. They found IL-38 can bind the IL-1RAPL1 receptor (also known as IL-1R8) and antagonize its effect on macrophage cytokine production stimulated by apoptotic cell culture conditions.[9] Moreover, IL-38 binding to IL-1RAPL1 was shown to suppress the feed-forward activation of IL-17-producing γδ T cells during psoriasiform skin inflammation.[17] Consequently, the aforementioned receptors have mostly been investigated as signaling pathways for IL-38[8,15,18] and it is worth inspecting these receptors and their most prominent ligands more closely.

3.1.3.1 IL-1R and ligands

The IL-1 sub-family consists of IL-1α, IL-1β and IL-33, which can bind different receptors of the IL-1R family. The most prominent receptor of the IL-1R family is the IL-1R1, which has been first characterized in 1985.[19] It belongs to the ligand binding receptors and is able to bind IL-1α, IL-1β, and IL-1Ra with similar affinity,[20] and reportedly also IL-38.[5,9] After binding an agonist, IL-1R3 as accessory chain also binds to the complex allowing the two TIR domains of IL-1R1 and IL-1R3 to approximate. This initiates intracellular signaling via MyD88 and in the end leads to the upregulation of NFκB or Activator protein 1 (AP-1) inducing an arsenal of inflammatory and immune stimulatory effects (reviewed in [21]).

IL-1α does not have a signal peptide and is mainly released upon cell death. Moreover, it contains an NLS and can act as a transcription factor.[22] It can bind the IL-1R1 as full-length precursor and as processed cytokine, both being biologically active.[23] The protease responsible for cleaving IL-1α is calpain-II.[24] Under necrotic circumstances, precursor IL-1α is released from cell lysates promoting a strong inflammatory stimulus via the IL-1R1.[25] This is characterized by an influx of neutrophils and monocytes.[26] Thus, IL-1α is referred to as an "alarmin" or danger associated molecular pattern molecule (DAMP).[23] Under apoptotic circumstances, IL-1α is translocated to the nucleus and is bound by DNA preventing it to exert its inflammatory signaling. Barrier cells like keratinocytes, endothelial cells and epithelial cells of mucus membranes constitutively express precursor IL-1α.[27] Therefore, it is believed that IL-1α is mainly involved in sensing the integrity of natural barriers and signaling possible damages. On B cells and monocytes, biologically active IL-1α is bound to the cell surface and therefore called membrane-IL-1α.[28,29] Even though IL-1α seems to be critical for early phases of inflammation, studies in IL-1α deficient mice indicated a greater role of IL-1β in the development of systemic responses (fever and acute phase proteins).[30] Furthermore, IL-1β seems to exert greater influence on IL-1α expression than contrariwise (reviewed in[31]).

IL-1β is commonly known for its strong pro-inflammatory effect and is primarily expressed in monocytes, macrophages, dendritic cells, NK-cells and B cells.[32] Different to IL-1α, IL-1β only functions in its processed variant. The process of IL-1β-activation is widely understood. Caspase-1 is the protease activating IL-1β intracellularly but must be activated itself first. Necessary to that end, a complex of intracellular proteins around NLR family pyrin domain containing 3 (NLRP3) was discovered and named the inflammasome.[33,34] The inflammasome is highly regulated and several mechanisms of induction have been described (reviewed in[31]). However, also NLRP3 independent mechanisms of IL-1β processing are known. This became evident as mice deficient of IL-1β can neither produce IL-6 or acute phase proteins nor develop fever upon sterile inflammation, while mice deficient of caspase-1 can.[35] For that purpose, different extracellular proteases (e.g. elastase, proteinase-3, MMP-9, granzyme A) have been identified to produce active IL-1β (reviewed in[31]). IL-1β is induced by bacterial products via

TLR stimulation but can also strongly induce itself in an autocrine loop.[36] IL-1β stimulates the immune response of various immune cells. It activates T-cells and together with IL-6, IL-23 and TGFβ, induces TH_{17}-cells.[37-39] Mice deficient of IL-1β do not spontaneously develop diseases.[31] However, their response to inflammatory challenges is altered. They have reduced inflammation upon zymosan induced peritonitis[35] and do not develop fever or produce acute phase proteins within 24 hours after application of a local irritant.[40]

Three mechanisms of antagonizing IL-1 cytokine signaling are of importance. First, a soluble form of the IL-1R (sIL-1R1) has been described and is believed to capture soluble IL-1 ligands to prevent their agonistic function on the IL-1R1.[41,42] Besides IL-1R2 is another receptor binding IL-1F cytokines in a membrane bound and a soluble form. Especially IL-1β is bound by IL-1R2 with high affinity. However, there is no downstream signaling as the receptor lacks the intracellular TIR-domain.[43,44] Consequently, IL-1R2 captures IL-1 cytokines and prevents IL-1R1 signaling, and is therefore referred to as decoy receptor.[45] IL-1Ra is the physiological competitive antagonist for the IL-1R1 and naturally controls IL-1 induced inflammatory reactions. It binds to the IL-1R1 and prevents the association of IL-1R3 thereby inhibiting downstream signaling.[46] The significance of IL-1Ra becomes evident in a rare genetic disorder named Deficiency of interleukin-1-antagonist (DIRA), where uncontrolled IL-1 dependent signaling leads to severe sterile multi-organ inflammations causing patients death at early ages.[47] The function of IL-1Ra has been used pharmacologically with great success. Anakinra is a generic IL-1Ra and is used in the treatment of various autoinflammatory diseases like rheumatoid arthritis, idiopathic pericarditis and others (reviewed in[48]). IL-1Ra does not bind to IL-1R2[44] being consistent with their purpose of preventing IL-1R1 signaling.

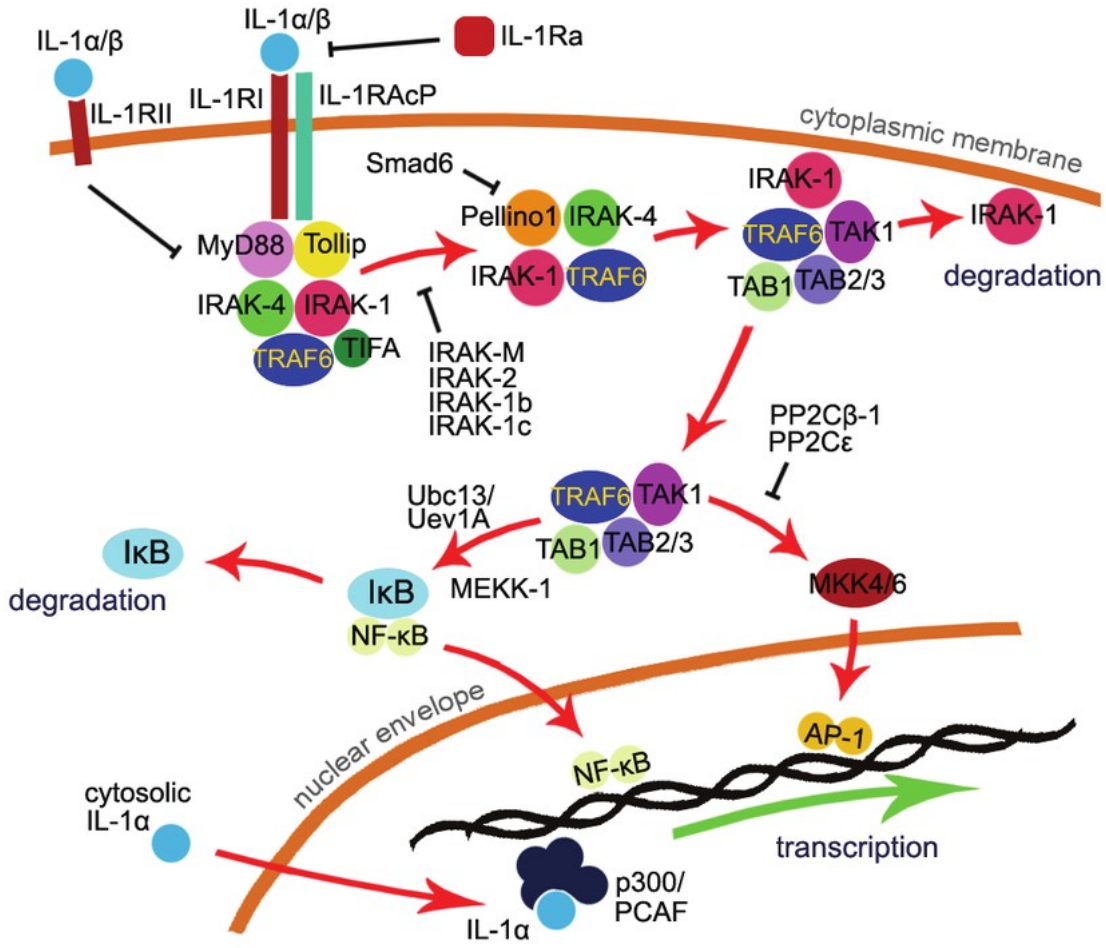


Figure 2: IL-1R1-signaling
IL-1α and IL-1β are able to bind the IL-1R1 and initiate downstream-signaling eventually leading to the upregulation of NFκB and AP-1. Two mechanisms of antagonizing IL-1R1-function are displayed. The IL-1Ra, which blocks intracellular signaling of the IL-1R1 and the presence of the IL-1R2, which captures IL-1α/β without inducing further signaling. Reprinted from Vicenová et al., Physiological Research, 2009.[49]

The ability of IL-38 to bind IL-1R1 with lower affinity than IL-1RA or IL-1β was described in 2001.[5] Boutet et al. found supernatant of IL-38 overexpressing THP-1 cells to exert an anti-inflammatory effect on synovial fibroblasts from rheumatoid arthritis patients stimulated with IL-1β.[50] In this scenario, IL-38 reduced IL-1β induced IL-6 production indicating a direct antagonistic effect through IL-1R1. Mora et al. also observed an inhibition of IL-1β induced IL-6 production in macrophages treated with truncated rh-IL38 while IL-6 production was not affected when macrophages were stimulated with lipopolysaccharides (LPS) as TLR4 ligand.[9] However, a direct biological effect of IL-38 through IL-1R1 signaling pathway has yet to be clarified.

3.1.3.2 IL-36R and ligands

The IL-36R binds all cytokines of the IL-36 sub-family, IL-36α/β/γ/Ra, and as previously described IL-38 as well. Similar to the IL-1R1, a trimeric complex is formed with the IL-1R3 leading to a downstream activation of NFκB and mitogen associated protein kinase (MAPK) and therefore a pro-inflammatory signal.[51,52] IL-36Ra has been proven to act as an antagonist on the IL-36R by binding to the receptor but inhibiting the recruitment of IL-1R3 and therefore acting anti-inflammatory.[53] IL-36 cytokines also seem to be involved in various immune responses. IL-36R is constitutively expressed by bone marrow-derived dendritic cells (BMDCs) and $CD4^+$ T-cells[54] pointing towards a critical role in the connection between innate and adaptive immune responses. Further it is expressed in keratinocytes, monocytes[52] and according to Penha et al. also on peripheral human B cells.[55] IL-36 agonists can stimulate $CD4^+$ T-cells to proliferate[55] and drive naïve T-cells into IFN-y producing Th_1-cells.[56] They are further known to enhance Th_{17} expansion and increase production of IL-23.[57]

IL-36 cytokines are mainly present in the skin, bronchial epithelium, dendritic cells and macrophages and seem to be important for homoeostasis in these tissues.[13,58,59] It is widely recognized that IL-36 cytokines are of importance in different autoimmune and inflammatory skin diseases. IL-36 agonists stimulate keratinocytes to secrete inflammatory cytokines and chemokines attracting immune cells and promoting inflammation.[60,61] In patients with psoriasis, high levels of IL-36 agonists have been detected in skin lesions.[62] Further, while IL-36R-deficient mice have reduced levels of IL-17 producing γδ-T-cells and develop less imiquimod induced skin lesions,[63] overexpression of IL-36α in keratinocytes directly leads to psoriasis like skin inflammation.[59] In addition, IL-36Ra deficiency in mice further increases psoriatic skin inflammation,[59] proving that the IL-36/IL-36Ra ratio plays a key role in this inflammatory skin disease. Similar findings have been made in psoriatic arthritis while the significance in rheumatoid arthritis is still unclear (reviewed in [64]).

To this day the IL-36R is discussed as most relevant receptor for IL-38 signaling. IL-38 was also added to the IL-36 sub-family due to the length of its precursor.[65] Van de Verdoonk showed IL-38 binding the IL-36R in vitro while carrying out similar antagonistic effects as IL-36Ra, particularly with similar kinetics. In

PBMCs treated with Candida albicans, IL-38 suppressed IL-17 and IL-22 production by Th17-cells. Furthermore, IL-38 was shown to inhibit IL-8 production by dendritic cells stimulated with IL-36γ clearly indicating IL-38 to act via the IL-36R. In these experiments, the binding affinity of IL-38 proved to be dose dependent with a maximum of 16.7 µg/ml.[16] Even though the IL-36R seems likely to be part of IL-38 signaling, few studies suggest otherwise. For one, knocking down IL-36R in human macrophages showed to have no effect on IL-6 production.[9] Furthermore, THP-1 cells have been shown to react to IL-38 treatment,[50,66] even though they are not known to express the IL-36R.[55,67] Therefore, the exact mechanism of IL-38 function is not fully explained by IL-36R dependent signaling only.

3.1.3.3 IL-1RAPL1

The IL-1RAPL1 is also known as IL-1R9 and was originally described as receptor involved in the pathophysiology of mental retardation.[68-71] It is generally known for its central nervous function as it interacts with neuronal calcium sensor-1 regulating exocytosis.[72,73] Initially the receptor was thought to be unable to bind any IL-1 cytokines[68,74] and was therefore referred to as an orphan receptor. IL-1RAPL1 is not able to induce NFκB production[70] but can activate the c-Jun N-terminal kinases (JNK) pathway.[75,76] Outside its known function in cerebral development, IL-1RAPL1 expression has been reported to be associated with asthma.[77] Mora et al. previously showed IL-1RAPL1 to be expressed by human monocyte derived macrophages and to be able to bind IL-38. In this scenario, truncated IL-38 acted as an antagonist, reduced IL-1RAPL1 dependent TH_{17} activation and inhibited IL-6 and IL-8 production through the JNK/AP1 pathway.[9] Interestingly, full length IL-38 had the opposite effect and increased IL-6 production by activating the JNK/AP1 pathway.[9]

Specific details about IL-1RAPL1 signaling and its role in immune responses require further investigations.

3.1.4 IL-38 in autoimmune diseases

In various association studies and functional studies, IL-38 has been correlated with different autoimmune and autoinflammatory disorders, especially rheumatic diseases. In comparison with healthy subjects, patients with rheumatoid arthritis

have increased IL-38 levels in serum as well as synovial membrane[11,78] and fluid.[79] This is also associated with increased concentrations of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6.[11] IL-38, IL-36Ra and IL-36 precursor cytokines, which are also increased in synovial membrane of rheumatoid arthritis patients,[78,79] all correlate with clinical severity of this autoimmune disease.[79,80] Simultaneously, IL-38 expression decreases after treatment of rheumatoid arthritis.[80] Moreover, single-nucleotide polymorphisms in the IL-38 gene of Korean patients have been shown to raise susceptibility of developing rheumatoid arthritis.[81] In the pathogenesis of rheumatoid arthritis, the predominance of Th1 and Th17 cells over regulatory T-cells is of importance.[82,83] Considering that IL-38 has been shown to suppress Th17-expansion,[16] it seems likely that the IL-38/IL-36R pathway plays a regulatory role in this particular disease. This has partly been confirmed in different mouse and in vitro models. Boutet et al. showed articular injection of an adeno-associated virus carrying IL-38 to decrease Th17 cytokines and clinical signs of inflammation in two different arthritis models in mice.[50] Further, medium from THP-1 cells overexpressing IL-38 was able to decrease IL-6, TNF-α and IL-23 production in macrophages and synovial fibroblasts from rheumatoid arthritis patients.[50] Fujimoto et al. showed IL-6 blockade to effectively inhibit development of collagen-induced arthritis in mice by suppressing TH17 responses.[84] Another autoinflammatory disease associated with IL-38 is psoriasis. Psoriasis is also strongly linked to an imbalance between Th1/Th17 and regulatory T cells[60,85] and as described above, IL-36 cytokines are well documented to heavily influence development of psoriatic lesions and clinical severity of this disease.[58,59,79,76,85] Normally, IL-38 is highly expressed in human skin, however in skin and peripheral blood of psoriatic patients, IL-38 expression is reduced.[17,79,86] Interestingly, in patients with the severe form of pustular psoriasis, IL-38 serum levels seem to be increased.[87] Polymorphisms in the IL-38 gene have also been correlated with psoriatic arthritis[88]. Animal studies have posed different biological importance of IL-38 in psoriasis models. Palomo et al. reported that IL-38 knockout mice did not show differences in progression and resolution of Imiquimod induced skin inflammation.[89] Han et al. however, reported IL-38 knockout mice to show delayed resolution of inflammation after Imiquimod induced psoriatic lesions. They observed increased IL-17 mediated skin inflammation in IL-38-knockout

mice which was reversable after treatment with recombinant IL-38. This effect was explained by IL-1RAPL1 dependent suppression of IL-17 producing γδ T cells.[17] Mercurio et al. found IL-38 to inhibit IL-36 precursor cytokine mediated inflammation in psoriatic skin. IL-17, IL-22 and IL-36γ promote strong inflammatory effects on human keratinocytes leading to their dedifferentiation and decreased expression of IL-38. Therefore, they postulated IL-38 might positively influence differentiation of keratinocytes and thereby reduce immune cell infiltration into the skin.[86]

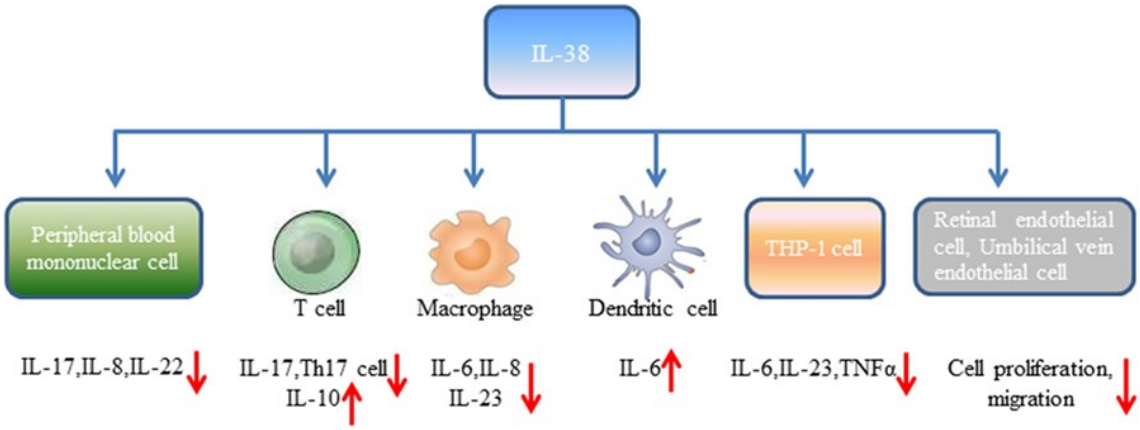


Figure 3: Overview of IL-38 affecting different immune cells
Displayed is an overview of effects IL-38 has on cytokine production of different cell types. Most of which depict an anti-inflammatory signal. The figure does not make the claim to be complete. Reprinted from Xu et al., Frontiers in immunology, 2018.[90]

Another autoinflammatory disease where Th17 dominance is essential part of its pathogenesis is systemic lupus erythematosus (SLE).[91] Intriguingly, different Studies have also drawn connections between IL-38 and this disorder. Rudloff et al. reported a protective role of endogenous IL-38 in patients with SLE. They showed IL-38 serum levels to be significantly increased in SLE patients compared to healthy controls. Further, they observed IL-38 to correlate with disease severity and activity as well as susceptibility to renal or central nervous complications.[92] Takeuchi et al. could not confirm these results in patients with juvenile onset-SLE.[93] Rudloff et al. further showed that the presence of IL-38 in sera of SLE patients was positively correlated with concentrations of the anti-inflammatory cytokine IL-10.[92] In vitro experiments knocking down IL-38 in human PBMCs revealed significantly increased production of SLE-related cytokines like IL-6, CCL2 and the tumour necrosis factor superfamily ligand APRIL in unstimulated PBMCs as well as after TLR7 or TLR9 stimulation.[92,94] In vivo experiments in

mice even showed that therapeutic use of IL-38 can ameliorate SLE symptoms and severity in affected animals.[95]

Intestinal bowel diseases (IBD) as Crohn´s disease (CD) and ulcerative colitis (UC) are also associated with dysregulated IL-36 signaling.[96-98] Therefore, the role of IL-38 is also of increasing interest in these disorders. Indeed, IL-38 is highly expressed in all intestinal layers of IBD patients.[99] In active UC, IL-38 expression was found to be higher compared to active CD.[96] Studies in CD patients showed that IL-38 levels together with IL-36α and IL-36γ are increased and correlate with IL-1β and IL-17A levels.[79] Animal studies using dextran sulphate sodium stimulated colitis confirmed increased expression of IL-38, IL-36α and IL-36γ in mice while IL-36β and IL-36Ra expression levels were not raised.[79,96] IL-36R knockout mice showed reduced intestinal inflammation in this colitis model indicating a crucial role of IL-36 cytokines in IBD.[96] As IL-38 most likely functions through IL-36R it is to assume that IL-38 is also of importance in IBD. Molecular details of how IL-38 might affect IBD remain to be elucidated.

Apart from the aforementioned disorders, increasing numbers of further diseases are reported to be associated with IL-38. Many of which are also connected with dysregulation in the IL-17 axis and elevated IL-36 precursor cytokines. In patients with primary Sjögren´s Syndrome, IL-38 and IL-36α expressions are increased in the salivary glands and IL-36α is elevated in the serum while correlating with disease activity.[100] Sjögren´s Syndrome is reportedly linked with dysregulated IL-23, IL-22 an IL-17 cytokine levels.[101] In Hidradadenitis suppurativa, a pustular skin disease, the IL-23/Th17 axis is also known as vital factor in disease emergence[102] and IL-38 is upregulated in its skin lesions appearing to exert a protective effect.[103] Different genome analyses have also reported further associations between IL-38 and multiple diseases. Polymorphisms in the IL-38 gene have been correlated with ankylosing spondylitis (AS) in Taiwanese Chinese and Chinese Han patients.[104,105] These findings were not confirmed in European AS-patients[106] but one study reported an association between IL-38 and non-ankylosing spondylarthritis.[107] Furthermore, a genome wide association study in more than 80.000 individuals indicated IL-38 being one of four proteins which serum levels correlate with those of C-reactive protein (CRP).[108] The others included IL-6R and are widely known for their role in inflammatory processes. In

East- and South Asian cohorts this correlation between IL-38 and CRP was confirmed.[109] Fang et al.[110] further reported a protective effect of IL-38 in sepsis. They found significantly increased IL-38 serum levels in adult and pediatric sepsis patients which correlated negatively with blood leukocytes and inflammatory cytokines like IL-6 and TNF-α. These findings were validated in polymicrobial sepsis models in mice. IL-38 levels in blood, lung, spleen and peritoneal lavage were clearly elevated after sepsis induction. In these models, the blockade of IL-38 with monoclonal antibodies disinhibited IL-6 and TNF-α and increased mortality in septic mice. Intriguingly, the injection of recombinant IL-38 two hours before sepsis induction had the opposite effect, improving survival rates of septic mice and decreasing measurable markers for organ failure and pro-inflammatory cytokine concentrations. Recently IL-38 has also been associated with lung diseases. Higher expression levels of IL-38 have been observed in idiopathic lung fibrosis[111] and correlate with poor prognosis in lung adenocarcinoma.[112] Moreover, latest data also suggest an association with myocardial infarction, as IL-38 is highly expressed in atheromatous plaques of coronary arteries[113,114] and is highly upregulated in ST-segment elevation infarction.[79]

In summary, IL-38 is still only a partly understood cytokine which seems to play a crucial role in the pathogenesis of different auto-inflammatory diseases and other disorders involving processes of inflammation. This has been pointed out by different association studies but so far only partly been confirmed through animal or in vitro studies. Most in vitro studies concerning IL-38 function have focused on immune cells, particularly T cells and macrophages. Even though, B cells are major participators in autoimmune disease, a potential interaction between IL-38 and B cells has not yet been investigated.

3.2 B cells

B lymphocytes are main effector cells of the adaptive immune system. They provide a unique, high affinity immune response and ensure long-term immunity against foreign pathogens. The molecular processes of B cell activation are therefore highly regulated and complex. Consequently, shortcomings in these regulatory mechanisms are causatively involved in a variety of diseases including malignancies and autoimmune diseases. In autoimmunity, B cells play a crucial role not only by producing autoantibodies, but also by presenting autoantigens, secreting inflammatory cytokines and other pathological mechanisms.[115]

3.2.1 Plasma cell development

The detailed processes of B cell development and plasma cell differentiation would go beyond the scope of this thesis. I would like to direct the reader to recent excellent reviews on that topic[116-118] and in this thesis focus on the most essential parts of plasma cell development and its regulatory mechanisms.

B cells evolve from haematopoietic stem cells in the bone marrow and are firstly termed Pro-B cells. The development of B cells in bone marrow depends on different transcription factors, particularly the paired box protein 5 (PAX5) defining commitment to the B cell lineage.[119] After successful rearrangement of the immunoglobulin heavy chain (IgH) in Pro-B cells, precursor B cells develop. These cells express the transmembrane form of μ IgH on their surface and in their Pre-B cell receptor (pre-BCR) which is an important checkpoint in B cell development allowing clonal expansion as well as rearrangement of the immunoglobulin light chain (IgL). Precursor B cells expressing IgM on their surface reach the second checkpoint initiating negative selection in which autoreactive cells are eliminated.[120-122] The cells surviving negative selection are IgM+ IgD- immature B cells and will exit the bone marrow and migrate to the spleen where they continue their maturation. They further develop through transitional stages (T1 and T2) becoming fully mature after a second negative selection step.[123] Transitional B cells partly reside in the marginal zone of the spleen to become non-circulating marginal-zone B cells.[124] The majority of T2 B cells however migrates to follicles in the spleen and lymph nodes to become circulating follicular B cells awaiting antigen encounter.[117] Follicular B cells, marginal-zone B cells and B1 B cells represent the group of mature naïve B cells

(mnBCs).[125] Phenotypically, they are defined as CD19+ CD20+ IgD+ CD27- B cells and with around 60% comprise the largest group of B cells found in peripheral human blood.[126,127] Antibody secreting cells can evolve from different B cell sub types depending on dose and location of antigen encounter.[117] The B cell subset most rapidly providing antibodies are B1 cells. These cells are predominantly found in corporal cavities and intestine and secrete natural IgM antibodies depicting a first line of defence against bacterial antigens.[128] After activation of B1 cells, they migrate to the spleen, lose CD5 expression and begin antibody secretion.[129,130] The spleen is indispensable in the formation and maintenance of these cells.[131] Another B cell subset quickly reacting to foreign antigens are marginal-zone B cells.[124,132] These cells mainly recognize antigens independently of T cell co-stimulation. After antigen encounter, they migrate from the marginal zone towards the red pulp of the spleen, proliferate vastly and differentiate into antibody secreting plasmablasts (PB). Plasmablasts are rarely found in peripheral human blood but are the predominant type of circulating antibody secreting cells. They are defined by a CD19+ CD27+ CD38+ phenotype. In contrast to terminally differentiated plasma cells (PC), plasmablasts highly proliferate and do not express CD138. Plasmablasts are commonly referred to as precursors of plasma cells.[116,133-137] In contrast to marginal-zone B cells, follicular cells are less sensitive towards antigen activation and respond to a smaller extent after LPS stimulation.[138] Follicular B cells usually receive T cell co-stimulation and form extrafollicular foci of plasmablasts and plasma cells after antigen encounter.[117] Antibody secreting cells originated from marginal-zone or follicular B cells do not have somatically hypermutated immunoglobulins. They supply a quick antibody response to foreign antigens, however, undergo apoptosis after a short period of time.[139]

After antigen encounter, follicular B cells can also establish a germinal center reaction which reaches its peak 10-14 days after immunisation.[117] To this end, antigen encounter and T cell help are also required.[140,141] Germinal centers provide a specialized environment for B cell proliferation and maturation, producing antigen specific long-lived plasma cells or memory B cells (MBCs).[142] The germinal center can be separated into dark and light zone. In the dark zone, proliferation and somatic hypermutation predominantly occur, while in the light

zone, affinity maturation of the BCR and stimulation by antigen specific T helper cells take place.[143] T cell help involves interaction between CD40 and the CD-40L and inducible T cell co-stimulator (ICOS) with its respective ligand.[144] Furthermore, different cytokines including IL-21 are important in driving plasma cell differentiation.[145] Follicular dendritic cells (FDC) are also crucial cells in germinal center reactions. They sequester and display antigens to the B cells in the light zone, which is fundamental for BCR signaling and affinity maturation.[146] B cells exiting the germinal center reaction are either plasma cells or memory B cells, both equipped with high-affinity BCRs and switched immunoglobulin classes[117]. In comparison to plasma cells, memory B cells have lower affinity to antigens, carry less mutations[147] and arise earlier in the immune response.[148] Memory B cells themselves do not produce antibodies but can rapidly respond to specific antigen encounter, proliferating quickly and differentiating into plasma cells.[149] A phenotypical marker for memory B cells is expression of CD27. Its expression is associated with plasma cell differentiation, antigen presentation and proliferation.[149-151] In peripheral human blood, CD27+ cells are almost exclusively considered as memory B cells which can be separated into non-switched IgD+ IgM+ and class-switched IgD- cells. A small proportion of switched memory B cells does not express CD27. These cells are still classified as memory B cells as they otherwise share surface phenotype and somatically mutated Ig V regions.[152,153]

The complex process of plasma cell differentiation is regulated by various transcription factors. First, PAX5 is the transcription factor defining B cell identity from early developmental stages in the bone marrow but must be downregulated later in order to allow plasma cell differentiation.[154,155] PAX5 induces transcription of several B cell specific genes including CD19 and IgA[156] and simultaneously inhibits transcription of X-box-binding protein 1 (XBP1), which is highly expressed in plasma cells. Further PAX5 supresses IgH and IgL expression as well as the immunoglobulin joining chain further explaining how PAX5 inhibits plasma cell differentiation.[157-159] PAX5 itself is activated by activation-induced cytidine deaminase (AID), an enzyme present in germinal centers and closely associated with somatic hypermutation and class-switch recombination in B cells.[160] PAX5 expression is maintained in memory B cells but lost during plasma cell

development.[161] When PAX5 decreases, upregulation of positive regulatory domain containing 1 (PRDM1) with its gene product BLIMP1 occur, signaling commitment to the plasma cell lineage and immunoglobulin production.[162] PRDM1 expression is dependent on the repression of another transcription factor named interferon regulatory factor 4 (IRF4). IRF4 is necessary for early proliferation of activated B cells and functions dose dependently.[163] While low expression levels affect germinal center B cell differentiation, higher levels cause PRDM1 upregulation and thereby initiate plasma cell differentiation.[164-166] PRDM1 then also increases IRF4 expression stabilizing plasma cell commitment and increasing XBP1 expression.[155] The latter is indispensable for plasma cells to produce large amounts of immunoglobulins[156] and leads to an increase in overall cell size, mitochondrial function and volume of the endoplasmatic reticulum.[167] In the germinal center, high expression of B cell lymphoma 6 (BCL6) is found[168] which is critical for germinal center formation.[169] One function of BCL6 is inhibiting PRDM1 expression to suppress plasma cell differentiation allowing the germinal center reaction to continue for an adequate time.[170,171] Therefore, decreasing BCL6 seems to be vital in induction of plasma cell development. In vitro, different signals as CD40L, immunoglobulin-specific antibodies[172] and crosslinking of the BCR[173] have been shown to reduce BCL6. On the other hand, PRDM1 activating signals as BCR stimulants[117] or TLR dependent mechanisms can also induce plasma cell differentiation. Particularly TLR4 ligands as LPS and TLR9 ligands as CpG-containing oligodeoxynucleotides are well documented to activate B cells and induce antibody production.[174,175] Moreover, stimulation by various cytokines is also an important mechanism controlling B cell differentiation. IL-6 alone,[176] the combination of IL-2 and IL-5[177] as well as IL-21[178] are known to enhance PRDM1 expression. Particularly, IL-21 strongly increases plasma cell generation by Signal transducer and activator of transcription 3 (STAT3) pathway activation[179,180] and promotes secretion of IgM, IgA and IgG by all mature B cells.[179,181] Production of IgE is particularly induced by IL-4 and IL-13 stimulation.[182,183]

After formation of plasma cells, they remain in the spleen for approximately two weeks, but can be detected in bone marrow for one year. Therefore, the bone marrow is seen as the primary niche for long-lived plasma cells enduring antibody

production.[184] Small proportions of long-lived plasma cells also reside in the spleen.[185] The exact mechanism of how plasma cells achieve the ability to survive for years is complex and not fully understood. One important part in this process is the reversal of pro-apoptotic signals which come along with plasma cell differentiation. Upregulation of PRDM1 directly inhibits the expression of anti-apoptotic mediators as BCL-2 family protein A1 while simultaneously inducing expression of apoptotic proteins.[186,187] Therefore, for plasma cells to sustain their own survival they must overcome these pro-apoptotic influences. In the bone marrow, stromal cells provide long lived plasma cells with different signals inducing long term survival. Among others, IL-6, B cell activating factor (BAFF), and the chemokine CXCL12 are relevant.[188-190]

3.3.3 Plasma cell differentiation in vitro

As explained above plasma cell differentiation is a highly regulated process occurring under specialized conditions and environment. Imitating this in vitro is challenging and requires special sequential stimulation. Several groups of researchers have successfully generated plasma cells antigen independently using different B cell subsets as origin. However, not all studies defined plasma cells by expression of CD138 but only CD20- CD38+ phenotype and switched Ig-classes.[191-199] Another limitation in many studies was the high proportion of spontaneous cell death.[190,193] As long-living plasma cells are almost exclusively found in the bone marrow, research concerning these cells requires in vitro models reliably producing them.

In 2007, Huggins et al. described a three-step culture system which was able to promote plasma cell differentiation of peripheral human blood B cells. As essential stimulants, CpG deoxynucleotide 2006 together with interleukins 2/6/10/15 and interferon-α (IFN- α) were used in different combinations during the culture process.[200] CPG DNA activates B cells and initiates differentiation through TLR9 signaling.[194] IL-10 enhances TLR9 signaling[201] and increases Ig-production[202-204] while IL-2 and IL-15 support proliferation.[200] IL-6 also strongly induces plasma cell differentiation as described above. Huggins´ group also added IFN-α, hepatocyte growth factor and hyaluronic acid in the final phase of cell culture, as they are known to enhance CD138 expression and plasma cell survival.[190,205,206] As CD40L suppresses later stages of plasma cell differentiation,

an anti CD40 antibody was also used in the second culture phase.[200] They observed that not only CD27+ B cells proliferated after initial TLR9 activation, but also CD27- mature naïve B cells (mnBC). However, mnBC only responded, when seeded under high density conditions. In the end they were able to create high proportions of Ig-producing, CD138+ plasma cells originating from both, mnBC and CD27+ peripheral human blood B cells.[200] In this in vitro differentiation process, progressive upregulation of CD27 and CD38 was observed. Simultaneously, IgD expression was lost and CD20 expression decreased. Two years after Huggins, Jourdan et al. further pursued the optimization of generating plasma cells in vitro. They applied small changes in the previous three-step culture system adding soluble CD40L as additional stimulant, imitating T cell help and yielding in higher initial amplification of the B cells.[207] CD40 stimulation in vivo is provided by activated T helper cells in the germinal center reaction.[208] Missing CD40 signals occurring in different disease like the X-linked hyper-IgM syndrome outline its importance in inducing somatic hypermutation, differentiation of MBCs and overall immunoglobulin production.[209-211] In vitro studies have shown CD40 stimulation to efficiently differentiate MBCs into short-lived IgG and IgM producing plasmablasts. Mature naïve B cells also respond to CD40 ligation by proliferating, isotype switching and increased CD27 expression, however only after longer duration of stimulation.[212]

With adding soluble CD40L to the aforementioned system of stimulants, Jourdan et al. were able to reliably produce CD138+ plasma cells from peripheral human blood MBCs and provided a detailed phenotypic and genotypic description of the cells during that process. As expected CD27 and CD38 upregulation accompanied plasma cell development, while CD20 expression was lost and surface HLA-II decreased. Production of immunoglobulins was raised throughout the differentiation process, intracellular IgM expression continuously decreased, while IgG expression increased between day 0 MBCs and day 10 plasma cells.[207]

In further studies Jourdan later described a new subset of B cells occurring during the process of in vitro differentiation from MBCs to plasma cells. These cells showed lower CD27 expression than initial MBCs with low or abundant CD20 and missing CD38 expression. Despite not expressing CD38, the cells were producing immunoglobulins at lower levels, showed plasma cell specific gene

expression, and later fully differentiated into plasmablasts and plasma cells. Therefore, these cells were termed pre-plasmablasts (Pre-PB).[213] Acre et al. confirmed an in vivo counterpart of CD38-, antibody secreting cells in human tonsils.[133] The biological relevance and exact regulation of pre-plasmablasts remains to be clarified.

3.4 Aim of this study

Our knowledge about IL-38 as the latest discovered cytokine in the IL-36 sub-family has steadily increased since its discovery in 2001. However, to this date its exact biological function, procession and signaling have not been fully characterized. In vitro studies on IL-38 indicate an anti-inflammatory property which is possibly exerted through three different receptors, IL-1R1, IL-36R and IL-1RAPL1. Moreover, various association studies have pointed out significant correlations and probably a protective role of IL-38 in different autoimmune disorders. Particularly inflammatory skin diseases seem to be affected by IL-38 being congruent with the fact that significant IL-38 expression has been described in keratinocytes. Interestingly, B cells have also been shown to express IL-38. However, to this day most studies concerning the biological function of IL-38 have focused on other cell types including T cells and macrophages, while possible interactions between IL-38 and B cells have not been investigated. Therefore, the physiological connection between IL-38 and B cells remains unclear. To this point it is unknown if IL-38 exerts an endogenous effect on B cells or if proliferating B cells secrete IL-38 to modulate their microenvironment by affecting other cell types. Further, B cells are reported to express the IL-36R, which is the most promising receptor for IL-38 signaling. The influence of IL-36R mediated signaling on B cells also remains unknown. Moreover, SLE is seen as classical B cell mediated autoimmune disease, in which autoantibodies crucially contribute to its pathogenesis. Intriguingly, a protective effect of IL-38 in SLE patients has been reported. Consequently, it seems logical to assume a potential connection between the novel IL-38 cytokine and B cell homeostasis.

In this study, our goal was to examine if exogenous IL-38 impacts B cells in the process of differentiating into antibody secreting cells. My task was first to establish an in vitro protocol allowing B cells to differentiate into antibody

secreting cells and secondly to investigate possible changes in cell phenotype, antibody production and gene expression induced by IL-38 treatment. Due to the heterogeneity of B cells and different fates of B cell differentiation, we decided to use B cells from peripheral human blood to simultaneously examine different B cell subsets.

4. Material and Methods

4.1 Material

4.1.1 Cells

Primary human PBMCs were isolated from buffy coats of anonymous donors obtained from DRK-Blutspendedienst Baden-Württemberg-Hessen, Institut für Transfusionsmedizin und Immunhämatologie, Frankfurt am Main. B cells were then isolated via negative selection using the Easy sep, B Cell sorting kit (Stemcell).

4.1.2 Stimulants and Inhibitor

Table 1: Stimulants and Inhibitors

Substance	Provider
Recombinant human soluble CD40-Ligand	Immunotools (Friesoythe, Germany)
Recombinant human IL-2	Immunotools (Friesoythe, Germany)
Recombinant human IL-6	Immunotools (Friesoythe, Germany)
Recombinant human IL-10	Immunotools (Friesoythe, Germany)
Recombinant human IL-15	Immunotools (Friesoythe, Germany)
Recombinant human IL-21	Immunotools (Friesoythe, Germany)
Recombinant human IL-38	Fraunhofer Institute IMTP (Frankfurt, Germany)
Recombinant human IFN-α	Immunotools (Friesoythe, Germany)
CpG oligonucleotide (ODN 2006)	Invivogen (Toulouse, France)
Accell SMARTpool Human IL1F10 siRNA	Dharmacon (Lafayette, USA)
siGENOME Non-Targeting siRNA Pool #1	Dharmacon (Lafayette, USA)

4.1.3 Antibodies

All antibodies were directed against human proteins and used for flow cytometry or ELISA.

Table 2: Antibodies

Antibody	Application	Dye	Provider	Dilution
B cell differentiation panel				
CD3	FACS	BV605	BD Biosciences GmbH (Heidelberg)	1:200
CD19	FACS	APC/Cy7	BioLegend (San Diego, USA)	1:100
CD20	FACS	Brilliant Violet 510	BioLegend (San Diego, USA)	1:200
CD27	FACS	Brilliant Violet 711	BioLegend (San Diego, USA)	1:100
CD38	FACS	BV421	BD Biosciences GmbH (Heidelberg)	1:200
CD45	FACS	Alexa Fluor® 700	BioLegend (San Diego, USA)	1:100
CD138	FACS	PE/Cy7	BioLegend (San Diego, USA)	1:50
IgD	FACS	FITC	BioLegend (San Diego, USA)	1:200
IgM	FACS	PE/Dazzle 594	BioLegend (San Diego, USA)	1:200
HLA-DR (MHC II)	FACS	APC	BioLegend (San Diego, USA)	1:100
B cell activation panel				
CD20	FACS	Brilliant Violet 510	BioLegend (San Diego, USA)	1:200
CD40	FACS	Brilliant Violet 605	BioLegend (San Diego, USA)	1:50
CD45	FACS	Alexa Fluor® 700	BioLegend (San Diego, USA)	1:100
CD69	FACS	PE/Cy7	BioLegend (San Diego, USA)	1:200
CD70	FACS	PE-CF594	BD Biosciences GmbH (Heidelberg)	1:200
CD80	FACS	BV711	BD Biosciences GmbH (Heidelberg)	1:200
CD83	FACS	PE	BioLegend (San Diego, USA)	1:200
CD86	FACS	FITC	Immunotools (Friesoythe, Germany)	1:100

HLA-ABC (MHC-I)	FACS	BV421	BD Biosciences GmbH (Heidelberg)	1:200
HLA-DR (MHC II)	FACS	APC	BioLegend (San Diego, USA)	1:100

4.1.4 Special reagents and kits

Table 3: Special reagents and kits

Reagent/kit	Provider
Iscove´s modified Dulbecco´s Medium	Thermo Fischer Scientific (Dreieich, Germany)
Accell siRNA Delivery Media	Dharmacon (Lafayette, USA)
Penicillin/Streptomycin	PAA Laboratories GmbH (Cölbe, Germany)
Human Holo-Transferrin Protein	R&D Systems (Minneapolis, USA)
Insulin from bovine pancreas	Sigma-Aldrich GmbH (Steinheim, Germany)
Biocoll separating solution	Biochrom GmbH (Berlin, Germany)
EasySep™ Human B Cell Isolation Kit	Stemcell (Vancouver, Canada)
Fc Blocking reagent	Miltenyi Biotec GmbH (Bergisch Gladbach)
Precision Count Beads	BioLegend (San Diego, USA)
Brilliant stain Buffer	BD Biosciences GmbH (Heidelberg)
Annexin-V-FITC	Immunotools (Friesoythe, Germany)
Propidium Iodide	Sigma-Aldrich GmbH (Steinheim, Germany)
Cell Proliferation Dye eFLuor® 670	eBioscience (San Diego, USA)
Albumin Fraction V	Carl Roth GmbH + Co. KG (Karlsruhe, Germany
Human IgA total Ready-SET-Go!®	eBioscience (Vienna, Austria)
Human IgG total Ready-SET-Go!®	eBioscience (Vienna, Austria)
Human IgM total Ready-SET-Go!®	eBioscience (Vienna, Austria)
peqGOLD RNAPure™	PeqLab Biotechnologie GmbH (Erlangen)

Maxima® First Strand cDNA Synthesis Kit	ThermoFisher Scientific GmbH (Dreieich)
Absolute™ qPCR SYBR® Green Fluorescein Mix	ThermoFisher Scientific GmbH (Dreieich)
Ethanol, Isopropanol, Chloroform	ThermoFisher Scientific GmbH (Dreieich)
DEPC-treated H_2O	ThermoFisher Scientific GmbH (Dreieich)
Nuclease free water	ThermoFisher Scientific GmbH (Dreieich)
Dimethyl sulfoxide	Carl Roth GmbH (Karlsruhe, Germany)
HEPES buffer solution	PAA Laboratories GmbH (Cölbe)

4.1.5 Buffer preparation

Table 4: Buffer preparation

Phosphate buffered saline (PBS)		**Erythrocyte lysis buffer**	
NaCl	137 mM	NH_4Cl	155 mM
KCl	2.7 mM	$NaHCO_3$	10 mM
Na_2HPO_4	8.1 mM	Na-EDTA	0.1 mM
KH_2PO_4	1.5 mM	pH	7.4
pH	7.4		

Annexin-V-buffer		**B cell sorting buffer**	
HEPES	10 mM	FCS	2%
NaCl	140 mM	EDTA	1 mM
$CaCl_2$	2.5 mM	In PBS	
Leukocyte washing buffer		**ELISA Wash Buffer**	
EDTA	2mM	Tween	0.05%
In 1 x PBS		in PBS	

4.1.6 Oligonucleotides

The primers for RT-qPCR were purchased at biomers.net GmbH (Ulm, Germany). Primer for IL-38 expression levels were ordered from Qiagen (Hilden, Germany).

Table 5: Human qPCR primers

Primer	Forward (5´→3´)	Reverse (5´→3´)
IL-38	RT² qPCR Primer Assay for Human IL1F10 (Qiagen)	
IL1R1	ATGAAATTGATGTTCGTCCCTGT	ACCACGCAATAGTAATGTCCTG
IL1RL2	TCCCGAAGAGTTGTGTTTTGG	TGAGTGTGTCAGTATGGCTTGA
IL1-RAPL1	ATGAAAGCTCCGATTCCACAC	TTTGGGCAAGGGAGTAATTTGT
PRDM1	AAGCAACTGGATGCGCTATGT	GGGATGGGCTTAATGGTGTAGAA
XBP1	CCCTCCAGAACATCTCCCCAT	ACATGACTGGGTCCAAGTTGT
PAX5	ACTTGCTCATCAAGGTGTCAG	TCCTCCAATTACCCCAGGCTT
IRF4	GCTGATCGACCAGATCGACAG	CGGTTGTAGTCCTGCTTGC
RPS27A	CTGGAAGATGGACGTACTTTGTC	CGACGAAGGCGACTAATTTTGC

4.1.7 Consumables

Table 6: Consumables

Material	Provider
Microplate 96-well, U-Bottom, sterile	Greiner bio-one GmbH (Frickenhausen)
ELISA microplates, high binding	Greiner bio-one GmbH (Frickenhausen)
PCR Plates	Bio-Rad Laboratories GmbH (Munich)
Eppendorf Cups (0.5 ml; 1.5 ml; 2 ml)	Eppendorf GmbH (Hamburg)
FACS tubes (5 ml)	Sarstedt AG & Co. (Nürnbrecht)
Pipet tips (10 µl; 100 µl; 1000 µl; 5000 µl)	Eppendorf GmbH (Hamburg)
Leucosep tubes	Greiner bio-one GmbH (Frickenhausen)
Plastic material (cell culture)	Greiner Bio-One GmbH (Frickenhausen)
Sterile filters (0,22 µm)	Millipore GmbH (Schwalbach)
BZO Adhesive Optical Seal Film	Biozym Scientific GmbH (Hessisch Oldendorf, Germany)

4.1.8 Instruments and software

Table 7: Instruments

Instruments	Provider
EasySep™ Magnet	Stemcell (Vancouver, Canada)

Apollo-1 LB 911 photometer	Berthold Technologies GmbH & Co. KG (Bad Wildbad)
Autoclave HV 85	BPW GmbH (Süssen)
Bacteria clean bench Hera guard	Heraeus GmbH (Hanau)
Heracell™ 240 incubator	Heraeus GmbH (Hanau)
Centrifuge 5415 R and 5810 R	Eppendorf GmbH (Hamburg)
LSRIIFortessa	BD Biosciences GmbH (Heidelberg)
Magnetic stirrer Combimag RCH	IKA Labortechnik GmbH & Co. KG (Staufen)
Mastercycler®	Eppendorf GmbH (Hamburg)
NanoDrop ND-1000	Peqlab Biotechnologie GmbH (Erlangen)
Neubauer improved counting chamber	Labor Optik GmbH (Friedrichsdorf)
Pipettes (10 µl, 100 µl, 1.000 µl)	Eppendorf GmbH (Hamburg)
Power washer 384	Tecan Deutschland GmbH (Crailsheim)
Pure water system Purelab Plus	ELGA LabWater GmbH (Siershahn)
Thermomixer compact	Eppendorf GmbH (Hamburg)
CFX96™ Real Time PCR Detection System	Bio-Rad (Hercules, USA)

Table 8: Software

Software	Provider
Citavi	Swiss Academic Software GmbH (Wädenswil, Schweiz)
FlowJo V10	FlowJo (Ashland, USA)
Microsoft Office	Microsoft Deutschland GmbH (München)
ND-1000 V3.2.1	Peqlab Biotechnologie GmbH (Erlangen)
Odyssey 2.1	Li-COR Biosciences GmbH (Bad Homburg)
Photo Read V1.2.0.0	Berthold Technologies GmbH & Co. KG (Bad Wildbad)
Prism 5	GraphPad Software (La Jolla, USA)

4.2 Methods

Buffer preparations are described in 4.1.6

4.2.1 PBMC separation and B cell isolation

Human PBMCs were isolated from buffy coats using Ficoll-Hypaque density gradient centrifugation. Two 50 ml Leucosep falcons were filled with 15 ml Biocoll separating solution and centrifuged shortly. Afterwards the buffy coats were equally divided into the falcons, filled up with Leukocyte washing buffer and centrifuged for 35 minutes at room temperature (440g, break off). PBMCs were then collected and washed twice with Leukocyte washing buffer. Red blood cells were removed by applying sterile Erythrocyte lysis buffer for 5 minutes, before washing again with Leukocyte washing buffer. The PBMCs were then counted using a hemocytometer and prepared in B cell sorting buffer at 50 x 106 cells/ml. The B cells were isolated using the EasySep™ Human B Cell Isolation Kit (Stemcell) which isolates B cells by negative selection while preventing pre-activation of the cells. The purity of B cells was greater than 95% confirmed by flow cytometry.

4.2.2 B cell culture

If not differently described, all experiments were performed in Iscove´s modified Dulbecco´s Medium (IMDM), containing 10% heat inactivated foetal calf serum (FCS), 100 U/ml penicillin, 100 mg/ml streptomycin, 50 µg/ml Transferrin and bovine Insulin (1:2000). The cells were plated in 200 µl in 96-well U-bottom plates at estimate of 500.000 cells/ml. The cells were incubated at 37° C with 5% CO2 in a Heracell™ 240 incubator (Heraeus GmbH).

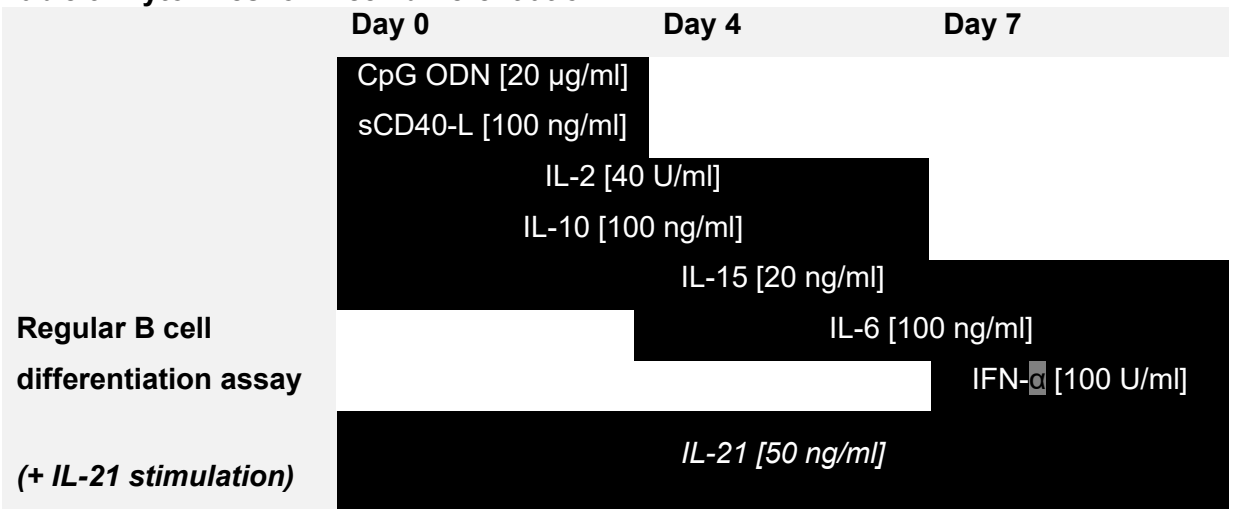
4.2.2.1 B cell differentiation assay

The B cells were cultured in a three-step culture system for a total of ten days with different cytokines being added as mentioned in

. On days 4, 7 and 10 supernatants were taken for further analysis, and cells were collected for FACS as well as for qPCR analysis. On days 4 and 7 the cells were washed with phosphate buffered saline (PBS) and plated in new medium without changing the concentration of cells per well. To one half of the cells rhIL-38 was added and compared to a control group not receiving exogenous IL-38. This treatment was given at 130 ng/ml on days 0 and 7 when seeding the cells and

repeated daily between day 3 and 6 at 65 ng/ml. In five separate experiments IL-21 was used additionally in both respective groups.

Table 9: Cytokines for B cell differentiation



4.2.2.2 B cell activation assay

In two separate experiments B cells were stimulated with CpG ODN [20µg/ml] for 24 h and then analyzed by flow cytometry. One group received ODN only, while two further groups also received IL-38. One of them at 65 ng/ml (IL-38low) and the other at 130 ng/ml (IL-38high).

4.2.2.3 Proliferation and cell death

The B cells were cultured and stimulated as described in 4.2.2.1 until day 7. Before seeding the cells, Cell proliferation dye eFluor 670 (eBioscience) was added according to the provider's instructions. To this end, a 5 µM solution of Cell Proliferation Dye eFluor™ 670 in PBS was mixed with cell suspensions and incubated for 10 minutes at 37°C in the dark. Afterwards the cells were washed and seeded as described above. On days 4 and 7, the cells were analyzed by flow cytometry. At the same time cell death was determined by Annexin-V/Propidium Iodide staining. For that purpose, the cells were harvested in FACS tubes and washed with Annexin-buffer. Fc-Blocking reagent was added at 1 µl in 100 µl Annexin-buffer for 15 minutes before incubating with 2 µl Annexin-V-FITC (Immunotools), 1 µl Propidium Iodide (SigmaAldrich) and anti CD27-Brilliant Violet 711 (BioLegend) for 20 minutes. After another washing step the cells were analyzed by flow cytometry.

4.2.2.4 IL-38 knockdown assay

Isolated B cells were incubated with 1 µM Accell SMARTpool Human IL1F10 siRNA (Dharmacon) in Accell siRNA Delivery Media (Dharmacon) containing 100 U/ml penicillin, 100 mg/ml streptomycin, 50 µg/ml Transferrin and bovine Insulin (1:2000). The cells were plated in 200 µl in 96-well U-bottom plates. After 24 h 20µl FCS was added to every well. The B cells were cultured for a total of ten days with different Cytokines being added as mentioned in **Table 9**. On day 4, 7 and 10 supernatants were taken for further analysis and cells were collected for FACS as well as for qPCR. On days 4 and 7 the cells were washed with PBS and plated in IMDM medium (see 4.2.2.1). As a non-targeting control Non-Targeting siRNA Pool #1 (Dharmacon) was used at 1 µM.

4.2.3 mRNA expression

4.2.3.1 RNA isolation from B cells

The B cells were harvested, washed and resuspended in 1 ml peqGold RNAPure™ (Peqlab Biotechnologie) and transferred to a 1.5 ml Eppendorf cup and then placed on ice for 5 minutes. 200 µl of chloroform were added, vortexed thoroughly and incubated at 4°C for 5 min. Subsequently, samples were centrifuged at 12,000g for 10 minutes to separate the RNA in an aqueous phase from phenol- and inter-phase. To precipitate RNA, 0.5 ml 2-propanol were added to the collected water phase, incubated for 20 minutes at -20°C and centrifuged again (12,000g, 15 min, 4°C). Precipitated RNA was washed twice with 75% ethanol in DEPC-H2O, dried (5 min, 70°C) and finally dissolved in 17 µl DEPC-H2O for 30 min at 55°C. RNA concentration was determined using the NanoDrop ND-1000 and optical density (OD) at 260 nm. An OD260 of 1 is equivalent to 40 ng/µl RNA.

4.2.3.2 Reverse transcription

To determine the amount of specific RNA transcripts, reverse transcription was performed according to the provided manual using Maxima® cDNA Synthesis Kit. In brief, 100-1000 ng of isolated mRNA were mixed with 4 µl 5 x reaction mix, 2 µl Maxima® reverse transcriptase and DEPC-H2O were added up to 20 µl. The reaction mix was incubated for 10 min at 25°C, 30 min at 50°C and finally 5 min

at 85°C for inactivation of the enzyme. The resulting cDNA was diluted to 1 µg/100 µl in DEPC-H2O.

4.2.3.3 Quantitative Real time polymerase chain reaction (qRT-PCR)

For qRT-PCR, 2 µl of cDNA were mixed with either 0.5 µl RT² qPCR Primer Assay for Human IL1F10 (Qiagen) or 0.25 µl of each forward and reverse primer (0.1 µM), 5 µl Absolute™ qPCR SYBR ® Green Fluorescein Mix and 2.5 µl Nuclease free H_2O. The mixtures were transferred to a qRT-PCR compatible plate. The plate was briefly centrifuged and sealed with BZO Adhesive Optical Seal Film (Biozym). Quantitative Real time PCR was carried out on a CFX96 system from Bio-Rad using following protocol.

Enzyme activation:	95°C, 15 min	
Denaturation:	95°C, 15 s	
Annealing:	60°C, 30 s	40 cycles
Extension:	72°C, 30 s	

To confirm specificity of the multiplication, a melting curve was created using the following program:

Denaturation:	95°C, 30 s
Starting temperature:	60°C, 30 s
Melting step:	60°C, 10 s, + 0.5°C per cycle, 80 cycles

Primer sequences are listed in **Table 5**. Target gene expression was calculated with ΔCt method [relative expression = $2^{-(Ct(target)-Ct(reference))}$] using expression of RPS27A as reference gene.

4.2.4 Immunoglobulin detection

Immunoglobulin concentrations in supernatants from B cell differentiation assays and IL-38 Knockdown experiments were determined using Human total Ready-SET-Go!® ELISA kits (eBioscience) for IgA, IgG and IgM. The ELISAs were performed according to the providers manual in 96-well high binding microplates (greinerBio). Briefly, the plates were covered with anti-human IgA/G/M capture

antibodies at 1:250 overnight at 4°C. After washing, the plates were blocked for 1 hour. Then the content was discarded and standard probes and samples were added in duplicates. For this, supernatants from cell culture were diluted 1:10 for IgM detection, 1:25 for IgA and 1:75 for IgG. Then seven-fold serial dilution was performed, and the plates were incubated at 37°C for 2 hours. Afterwards plates were washed, a horseradish peroxidase- (HRP) conjugated antibody was added at 1:250 for 1 hour, washed again and substrate solution was applied for 15-20 minutes. The reaction was then stopped with 2M H_2SO_4 and optical density was determined at 450/560 nm using Apollo-1 LB 911 photometer (Berthold Technologies GmbH & Co. KG). Final Ig concentrations were calculated via Microsoft Excel.

4.2.5 Flow Cytometry

Single cell suspensions were treated with 2 µl FcR blocking reagent (Miltenyi Biotec) in 50 µl PBS/BSA 0.5% for 10 minutes followed by a 20-minute staining with the antibodies as listed in Table 2. The antibodies were premixed in 50 µl Brilliant stain Buffer (BD Biosciences GmbH). All incubation steps were performed on ice and in the dark. All samples were then washed with PBS/BSA 0.5% and centrifuged (500 x g, 5 min, 4°C) followed by resuspending the cells in FACS flow. Samples were acquired using an LSR Fortessa flow cytometer (BD Biosciences) and data were analyzed with FlowJo software.

4.2.6 Statistical analysis

All statistical tests were performed using Prism 5 (GraphPad Software). The results were first tested for normal distribution via the Kolmogorov-Smirnow-Test. In case of gaussian distribution a paired t-test was used for analyzing results between control group and treatment group. In case gaussian distribution was not given, the Wilcoxon matched-pairs signed rank test was performed. If testing for normal distribution was not possible due to low number of replicates, a gaussian distribution was assumed and paired t-test was performed. Throughout, a threshold of significance was applied for $p < 0.05$.

5. Results

5.1 IL-38 does not affect B cell activation

We were initially interested to find out if IL-38 affects B cell activation. First, we tested if we could activate B cells in vitro using only CpG-ODN as TLR9 stimulant. Hence, we collected peripheral human blood B cells by magnetic depletion of non-B cells using the EasySep™ Human B Cell Isolation Kit, which, compared to positive selection, avoids pre-activation of B cells. Next, the cells were stimulated with CpG-ODN for 24 h. Afterwards, the expression levels of specific B cell surface proteins (CD40, CD69, CD70, CD80, CD83, CD86) were determined by flow cytometry. These markers are quickly upregulated as B cells become activated.[214,215] As displayed in **Figure 4**, the cells responded to CpG-ODN stimulation and showed increased expression levels of all tested markers with the exemption of CD70 (not shown). Next, we examined if exogenous IL-38 altered this early B cell activation upon CpG-ODN stimulation. Therefore, one group of cells was additionally treated with rh-IL-38 at 65 ng/ml (IL-38 low) or 130 ng/ml (IL-38 high). In two experiments, we did not observe an impact of exogenous IL-38 on the expression levels of any B cell activation marker. Therefore, we decided not to perform further experiments concerning the influence of IL-38 on early B cell activation but focussed on the process of differentiating peripheral human blood B cells into antibody secreting cells.

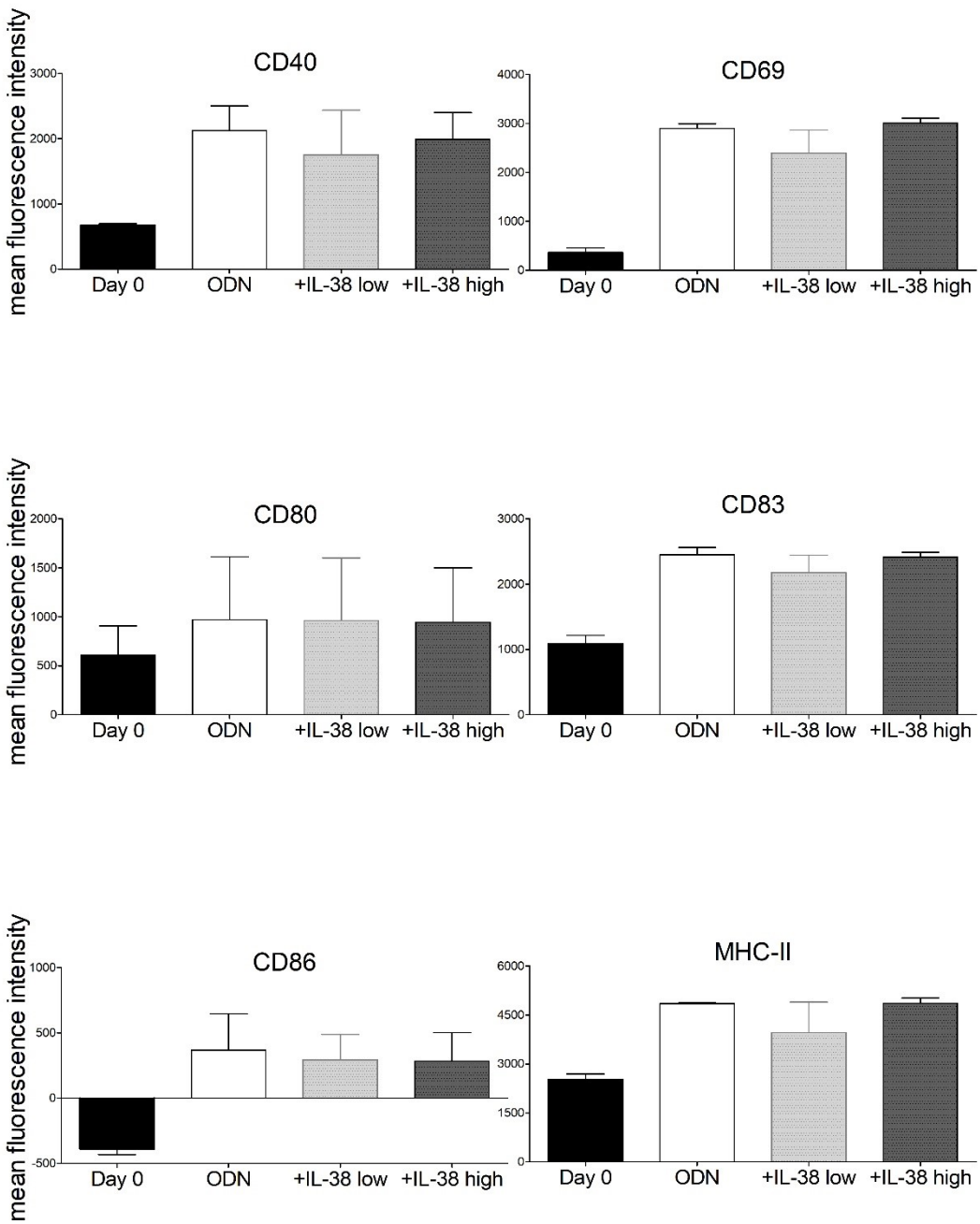


Figure 4: B cell activation after 24 h CpG-ODN stimulation
B cells from peripheral human blood were cultured with CpG-ODN (20 µg/ml) for 24 h. The cells were additionally treated with rhIL-38 at 65 ng/ml (+IL-38 low) or 130 ng/ml (+IL-38 high). Before and after stimulation, expression levels of B cell activation markers were determined by flow cytometry using anti-human mABs. n = 2.

5.2 Impact of IL-38 on B cell differentiation

To investigate the influence of exogenous IL-38 in the differentiation of naïve or memory B cells to plasma cells, we needed to establish a reliable protocol allowing us to differentiate B cells from peripheral human blood in vitro.

5.2.1 Establishing a plasma cell differentiation protocol

B cells were isolated from human buffy coats of anonymous donors by magnetic depletion of non-B cells using the EasySep™ Human B Cell Isolation Kit, which, compared to positive selection, avoids pre-activation of B cells. Afterwards, the isolated B cells (purity >95%) were characterized by flow cytometry (**Figure 5**).

In flow cytometric analysis, dead cells were first removed by characteristic physical properties (FSClow, SSCint/high). After excluding non-B cells by CD19- CD20- phenotype, the two largest populations of peripheral human blood B cells were divided by CD27 and IgD expression (**Figure 5A**). A mean of 69.64% of all B cells were IgD+ CD27- cells and therefore termed mature naive B cells. A mean 21.57% the cells were expressing CD27. In this CD27+ sub-group, almost all cells were CD20+ CD38- (**Figure 5B**). These cells were also expressing MHC-II and consequently described as memory B cells. They represented the second largest population of cells on day 0 with 20.40% of all B cells.

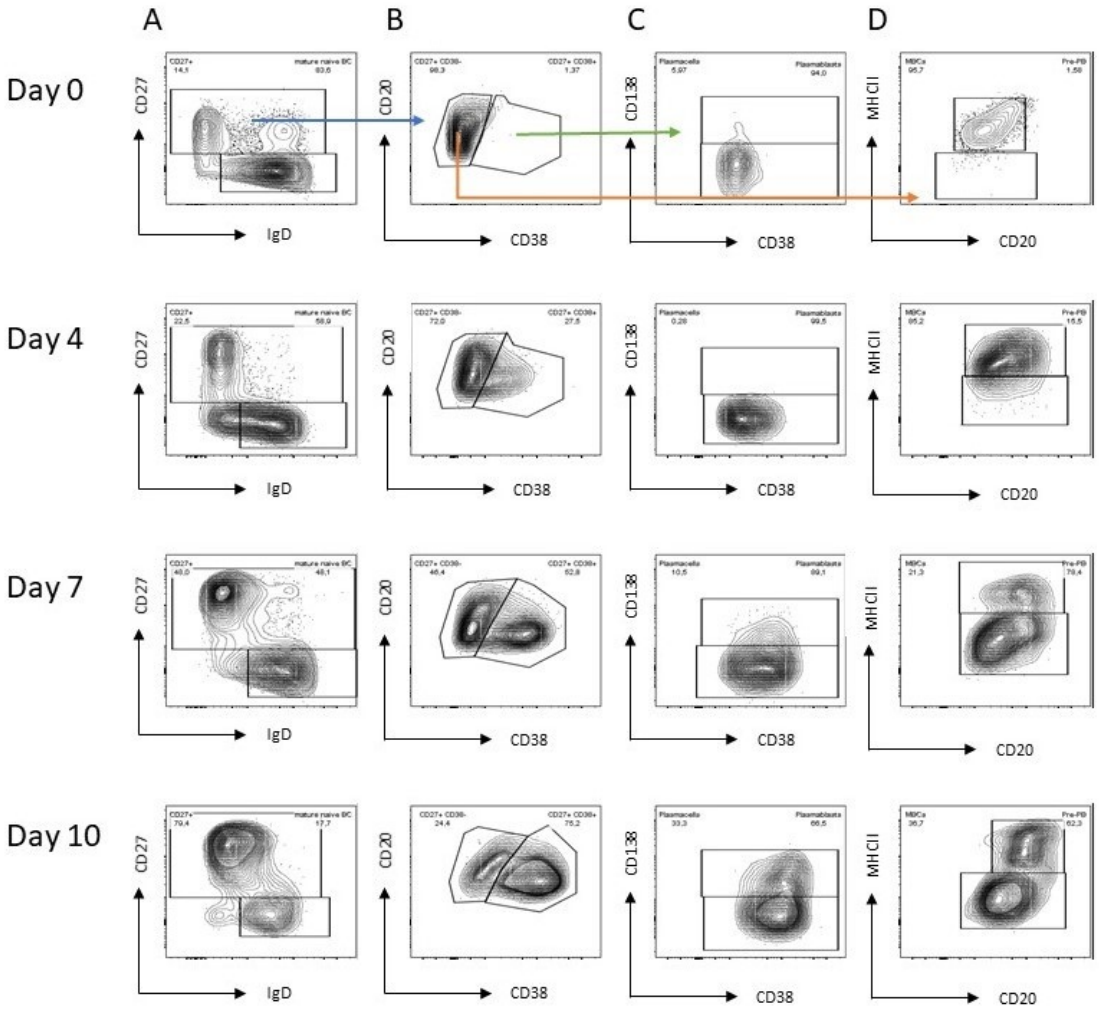


Figure 5: Gating strategy of B cell differentiation assay
B cells from peripheral human blood were cultured in a three-step culture system. On day 0 the cells were stimulated with CpG-ODN (20 µg/ml), sCD40-L (100 ng/ml), IL-2 (40 U/ml), IL-10 (100 ng/ml) and IL-15 (20 ng/ml) for 4 days. On day 4, the cells were washed and cultured with IL-2 (40 U/ml), IL-6 (100 ng/ml), IL-10 (100 ng/ml) and IL-15 (20 ng/ml) until day 7. After washing, the cells were cultured for three additional days with IL-6 (100 ng/ml), IL-15 (20 ng/ml) and IFN-α (100 U/ml). At each culture step, the cells were harvested and analyzed by flow cytometry using fluorochrome conjugated mABs to determine cell subsets. (**A**) B cells after removal of dead cells and non-immune cells. (**B**) CD27+ cell population gated from A (blue arrow). (**C**) CD27+ CD38- cells gated from B (green arrow). (**D**) CD27+ CD38+ cells gated from B (orange arrow). The shown experiment is representative for six individual experiments.

Next, B cells were initially stimulated with CpG-ODN (20 µg/ml), sCD40-L (100 ng/ml), IL-2 (40 U/ml), IL-10 (100 ng/ml) and IL-15 (20 ng/ml) for 4 days to induce proliferation and activate differentiation into antibody secreting cells. On day 4, 48.29% were still defined as naïve B cells, while 32.40% had acquired CD27+ phenotype. Of these CD27+ cells, 75.35% were CD20+ CD38- cells, which could be further separated into MHC-II+ memory B cells and another MHC-II- cell population (**Figure 5D**). This population was termed pre-plasmablasts (Pre-PB), which are a subset of cells in the differentiation of memory B cells to plasma cells and are characterized by low CD20 and loss of MHC-II expression. 54.15% of D4 CD27+ cells were defined as memory B cells and 21.26% Pre-PB. In contrast to unstimulated B cells, CD38 activation also became visible on day 4 (**Figure 5B**), which is an important phenotypic feature in the development of antibody producing cells. CD38+ cells were only detected in the group of CD27+ cells and comprised for 24.42% of D4 CD27+ cells. All D4 CD27+ CD38+ cells were negative for plasma cell marker CD138 and therefore termed plasmablasts (PB).

In a second culture step, the cells were stimulated with IL-2 (40 U/ml), IL-6 (100 ng/ml), IL-10 (100 ng/ml) and IL-15 (20 ng/ml) for 3 days to further induce differentiation towards PC. On day 7, 37.42% of all B cells were mature naïve cells and 53.82% CD27+ cells. Even though the overall CD27+ cell population increased, the percentage of memory B cells further decreased to 18.79% of D7 CD27+ cells. Pre-PB comprised for 41.93% of D7 CD27+ cells. The proportion of CD38+ cells also rose, making up 38.44% of the CD27+ population and for the first time, CD138+ plasma cells were detected as 1.48% of CD27+ cells and 0.76% of all B cells. For the final 3 days of in vitro differentiation, the cells were

stimulated with IL-6 (100 ng/ml), IL-15 (20 ng/ml) and IFN-α (100 U/ml) to increase plasma cell differentiation and survival.

At the end of the in vitro differentiation protocol, a mean 24.87% of all B cells were still defined as CD27- IgD+ mature naïve B cells. The percentage of CD27+ cells did not further increase between day 7 and day 10 reaching a mean 53.10% of all B cells (**Figure 6A**). The fraction of CD38+ cells however increased to 51.45% of D10 CD27+ cells. In general, focussing on CD27+ CD38+ cells, we observed a constant increase in CD38+ cells between day 0 and day 10 and in the group of cells highly expressing CD38, CD138+ plasma cells developed (**Figure 5C**). Therewith, the percentages of PB and PC also rose between day 7 and day 10, reaching 43.72% and 7.67% of D10 CD27+ cells (**Figure 6B**). Interestingly, within the CD27+ CD38- group of cells, the percentage of memory B cells was not further reduced after day 7 (22.60% of D10 CD27+), while Pre-PB decreased clearly to 25.62% of D10 CD27+ cells. This may indicate, that in this protocol of PC differentiation, after day 7 no more memory B cells were stimulated to differentiate into PC, while existing Pre-PB were induced to develop into plasma cells.

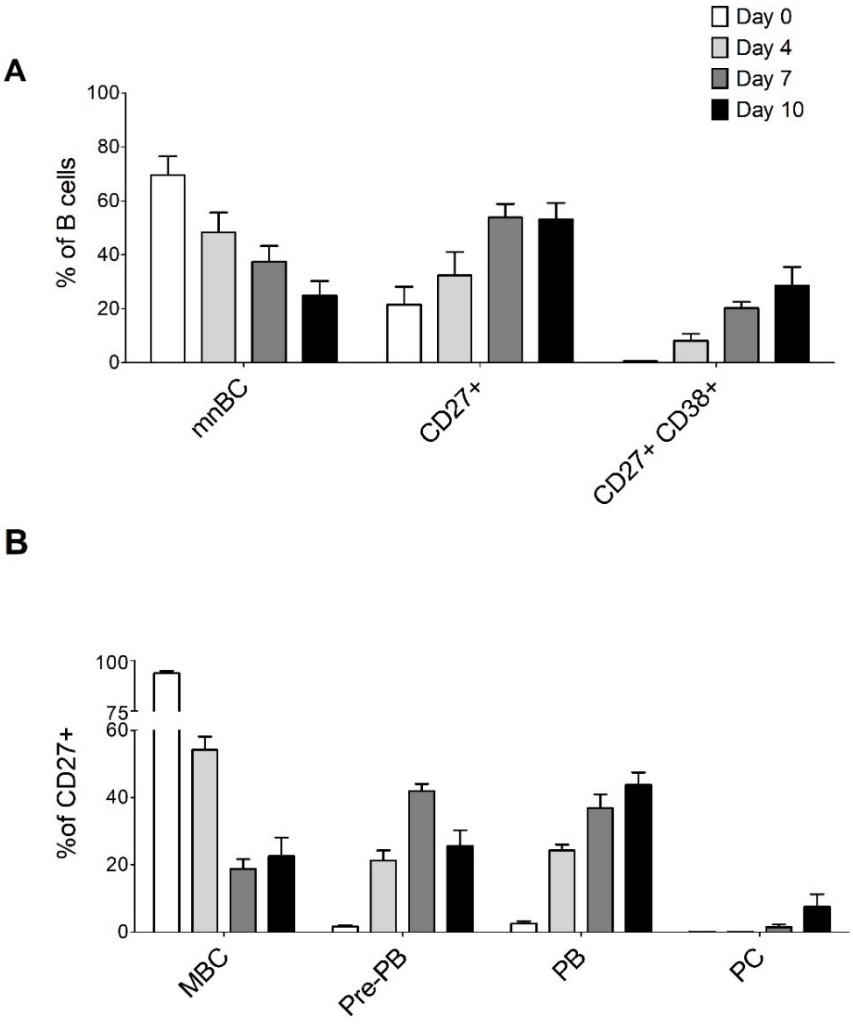


Figure 6: Successful generation of plasma cells from peripheral human blood B cells
B cells from peripheral human blood were cultured in a three-step culture system as indicated above. At each culture step, the cells were harvested and analyzed by flow cytometry, using fluorochrome conjugated mABs to determine cell subsets. Data are mean percentages with SEM of all B cells (**A**) and of CD27+ cells (**B**). n = 6. mnBC: mature naïve B cells, MBC: memory B cells, Pre-PB: preplasmablasts, PB: plasmablasts, PC: plasma cells

5.2.2 IL-38 receptors are present on differentiating B cells

As described in **3.1.3**, there are few receptors known to bind IL-38 which have partly been reported to also be present on human B cells. Therefore, we first wanted to confirm the presence of IL-38 binding receptors on the B cells at the level of mRNA-expression. We focused on the three most promising receptors IL-1R1 (IL1R1), IL-36R (IL1RL2) and IL-1RAPL1. Of these, the IL-36R was detectable in all 6 experiments and all samples of peripheral human blood B cells.

IL-1R1 and especially IL-1RAPL1 expression was not as reliably detectable. IL-1R1 expression was evident in 5 donors, IL-1RAPL1 in three. Therefore, findings for IL-1RAPL1 expression lack statistical profundity. During the B cell differentiation assay IL-1RAPL1 and IL-36R expression underwent only small mean fold changes. While IL-1RAPL1 seemed to be slowly but steadily upregulated throughout the differentiation process, IL-36R expression remained on a similar level between day 4 and 10. IL-1R1 mRNA however was strongly upregulated on day 4, with a mean fold change of 130 in the control group. Afterwards its expression level steadily decreased towards day 10 (**Figure 7**).

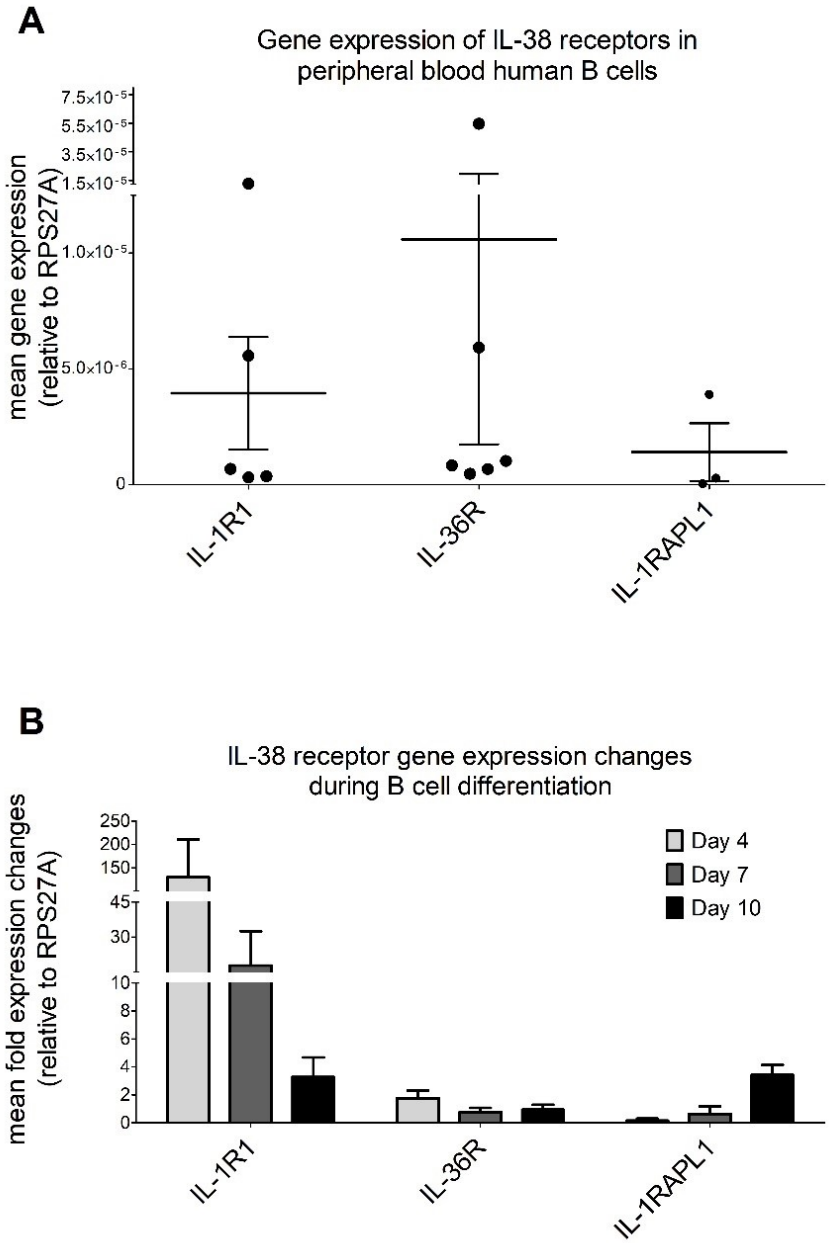


Figure 7: IL-38 receptor gene expression during B cell differentiation
B cells from peripheral human blood were cultured in a three-step culture system as indicated above. At each culture step, cells were harvested and washed. RNA expression levels were determined by Real time qPCR. Data are mean expression levels with SEM relative to RPS27A (**A**) in peripheral human blood B cells and (**B**) throughout B cell differentiation. n = 6 (IL-36R), n = 5 (IL-1R1), n = 3 (IL-1RAPL1).

5.2.3 Exogenous IL-38 reduces CD38 expression in differentiating B cells

After establishing a protocol allowing us to differentiate naïve and memory B cells into plasma cells, we wanted to test, if exogenous IL-38 would influence this differentiation process. Therefore, after isolating B cells from peripheral human blood, we separated the cells in two groups. The control group of cells, referred

to as “normal” were cultured as described above, while the treatment group, referred to as “+IL-38” received additional rhIL-38 into their medium. IL-38 was added at 130 ng/ml on day 0 and 7 and daily at 65 ng/ml between day 3 and 6. The double concentration of IL38 was used on days 0 and 7 as a daily application for ten consecutive days would not have been feasible.

Comparing proportions of different cell subtypes in the control group and the IL-38 treated cells during B cell differentiation, we did not find significant differences on day 4. This was apparent when analyzing percentages related to all B cells in culture at this time point as well as percentages of CD27+ cells (data not shown). On day 7 however, we observed a highly significant difference in CD38 expression in both experimental groups as lower percentages of cells were expressing CD38 when treated with exogenous IL-38 **(Figure 8**). In the IL-38 treated group the CD27+ CD38- cells comprised for a mean 37.62% of all B cells compared to 33.43% in the control group ($p = 0.0008$). The percentage of Pre-PB as a subgroup of CD27+ CD38- cells was also increased in the IL-38 treated cells (25.87% compared to 22.56%, $p = 0.0184$). CD27+ cells in total and CD27+ CD38+ cells did not differ significantly (**Figure 8A**). To further examine if the CD38 expression differences evolve from the population of CD27+ cells, we analyzed this group separately (**Figure 8B**). Here, we also found the CD38- fraction of cells to be elevated through IL-38 treatment with 66.09% compared to 61.23% in the control ($p = 0.0497$). Vice versa, the percentage of CD38+ cells was higher in the control group with 38.44% compared to 33.68% of D7 CD27+ cells ($p = 0.0468$). Also, the percentage of plasmablasts, being the largest subgroup of CD27+ CD38+ cells, differed significantly. In the control group, 36.89% of D7 CD27+ cells were defined plasmablasts, compared to 32.43% in the IL-38 group ($p = 0.0405$). However, no difference in plasma cells was evident on this day.

On day 10 of the differentiation process, the changes in CD38 expression were also visible. In the IL-38 treated cells, the CD27+ CD38- group made up for a mean 30.42% of all B cells compared to 24.38% in the control group ($p = 0.0341$). The share of Pre-PB of all B cells was also different with 17.81% in the treatment group and 12.63% in the control ($p = 0.0235$) (**Figure 8C**). Analyzing cell subsets in the group of CD27+ cells, the differences observed on day 7 in CD38- and in CD38+ cells closely failed statistical significance on day 10 ($p = 0.0529$; $p = 0.0639$) (**Figure 8D**). As we had observed on day 7, the differences in CD38 expression had no visible effect on the share of plasma cells at the end of the culture process. In summary, IL-38 mainly reduces CD38 expression in the group of CD27+ cells on days 7 and 10 of the differentiation process (**Figure 8E**).

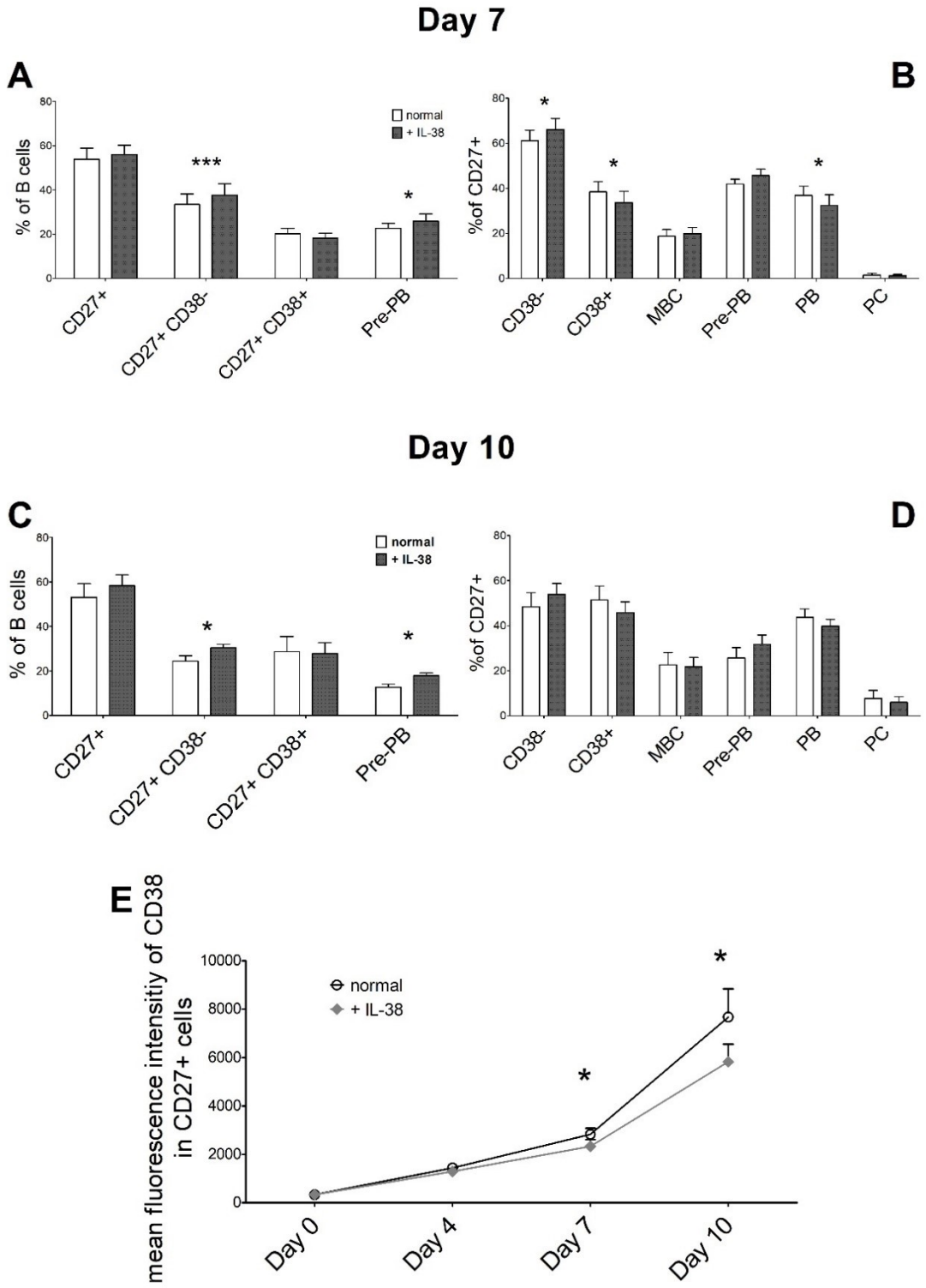


Figure 8: Exogenous IL-38 reduces CD38 expression on days 7 and 10
B cells from peripheral human blood were cultured in a three-step culture system as indicated above. The + IL-38 group received rhIL-38 at 130 ng/ml on day 0 and 7 and 65 ng/ml daily between day 3 and 6. At each culture step, the cells were harvested and analyzed by flow cytometry, using fluorochrome conjugated mABs to determine cell subsets. Data are mean percentages with SEM of all B cells (**A, C**) and of CD27+ cells (**B, D**). n = 6. *p < 0.05, ***p < 0.001. mnBC: mature naïve B cells, MBC: memory B cells, Pre-PB: preplasmablasts, PB: plasmablasts, PC: plasma cells

5.2.4 IL-38 treatment reduces early antibody production

As we had established a protocol to differentiate naïve and memory B cells into plasma cells and had observed cell phenotypic changes by IL-38 treatment during the culture process, we next wanted to examine B cell function by testing immunoglobin production. Hence, we collected supernatant from the cell culture on days 4, 7 and 10 and analyzed the concentrations of IgG, IgM and IgA. We chose not to test for IgE, as we did not stimulate the cells with IL-4 and therefore were not expecting the cells to produce IgE in relevant amounts. All tests were carried out on 96-well high binding microplates using Human total Ready-SET-Go!® ELISA kits according to the providers instructions. Supernatants were used at optimal dilutions in PBS, 1:10 for IgM, 1:25 for IgA and 1:75 for IgG. In both experimental groups, all tested immunoglobulins could be detected on day 4 and increased strongly up to day 7. IgM and IgA concentrations decreased between day 7 and 10, while IgG levels further increased. To correct possible differences in total Ig concentrations to be caused by different numbers of living cells in culture, we also normalized Ig production to the number of living cells at the time of analysis. Investigating Ig production per cell and day, we observed that IgG and IgM secretion increased constantly from day 4 to day 10, while IgM reached its maximum on day 7, before decreasing again in both experimental settings. As expected in early B cell immune responses, IgM levels in ng/ml and in pg/cell/day were higher compared to IgA or IgG at each time points. Comparing the IL-38 treated cells with the control group, we observed significant differences in total Ig concentrations in all tested subclasses on day 4 (**Figure 9**). IgM concentrations in supernatants of D4 cells reached a mean of 8763 ng/ml in the control group and 7071 ng/ml in the IL-38 treated cells (**Figure 9A**) ($p = 0.0313$). However, this difference was not observed when analyzing per cell production (**Figure 9B**). On day 7 there was no significant difference in either category, but interestingly day 10 cells clearly produced more IgM per day, when they received IL-38 with 49.82 compared to 38.16 pg/cell/day in untreated cells ($p = 0.0076$). As expected, total IgG concentrations were lower than IgM levels on day 4. Comparing the control group and IL-38 treated cells, we found significantly higher IgG concentrations in supernatants of the control group on day 4 with 253.99 ng/ml compared to 195.73 ng/ml ($p = 0.0002$) (**Figure 9C**). However, analyzing D4 IgG production per cell and day did not confirm a statistically significant difference even though a

tendency was observed (p = 0.0871) (**Figure 9D**). On day 7, total IgG concentrations also seemed to be decreased in the IL-38 treated cells (2406 ng/ml compared to 2165 ng/ml), but closely missed statistical significance (p = 0.0527). The concentration of IgG in supernatant increased further to day 10, reaching 3013 ng/ml in control cells and 2393 ng/ml in treated cells (not significant). The production per cell also increased further reaching around 21 pg/cell/day in both respective groups. D4 IgA concentrations were also lower in IL-38 treated cells with a mean of 463,2 ng/ml compared to 541.1 ng/ml in the control group (p = 0.0333) (**Figure 9E**). This difference was also visible analyzing the IgA production per cell and day with 0.41 compared to 0.45 pg/cell/day (p = 0.0235) (**Figure 9F**). For days 7 and 10, no differences in IgA production became evident.

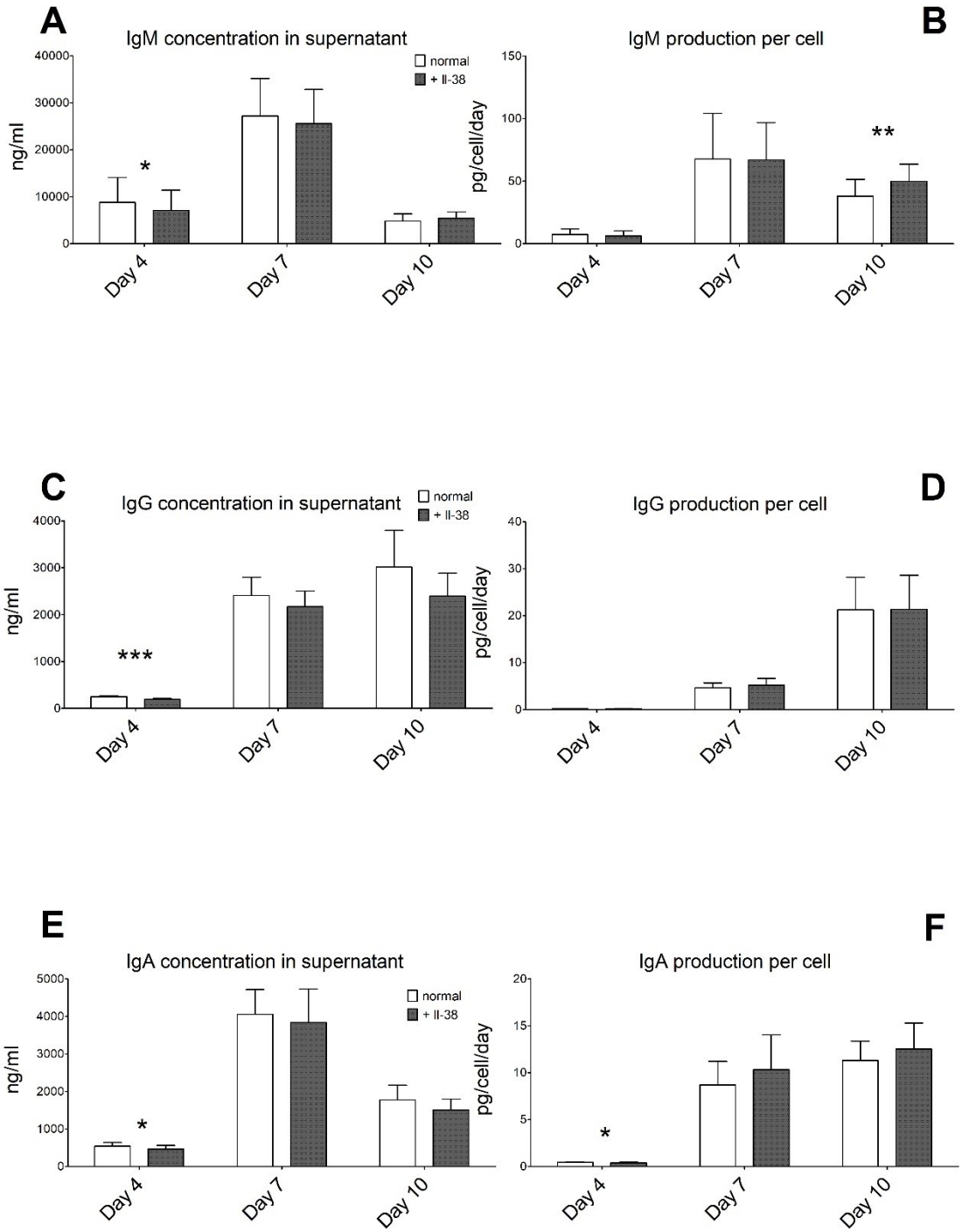


Figure 9: Impact of exogenous IL-38 on immunoglobulin production
B cells from peripheral human blood were cultured in a three-step culture system as indicated above. The + IL-38 group received rhIL-38 at 130 ng/ml on day 0 and 7 and 65 ng/ml daily between day 3 and 6. At each culture step, supernatant was obtained and analyzed using Human total Ready-SET-Go!® ELISA kits for IgA, IgG and IgM. Data are mean immunoglobulin concentrations in cell culture supernatants (**A, C, E**) and immunoglobulin production per cell and day (**B, D, F**) with SEM. n = 6. *p < 0.05, ***p < 0.001.

5.2.5 Impact of exogenous IL-38 on plasma cell specific gene expression

To further study the influence of IL-38 on B cells, we analyzed genes, which are known to play specific roles during B cell differentiation and plasma cell development. As described in **3.2.1**, B cells differentiating towards plasma cells downregulate PAX5 which consequently increases the expression of PRDM1 (Blimp1). In our experiments we observed an increase in PAX5 levels from day 0 to day 4 by around 50% in both experimental groups (**Figure 10A**). After day 4, the expression levels constantly decreased. On day 10 the mean fold change compared to day 0 B cells was 0.224 in the control group and 0.351 in the IL-38 treated cells (not significant). PRDM1 was also strongly upregulated after stimulation. On day 4, the expression levels in both groups were around 40 times higher compared to day 0 but did not increase further during the differentiation process (**Figure 10B**). Similar results were found for XBP1 (**Figure 10C**) which was also clearly upregulated on day 4 but remained on this level without significant differences between control and treatment group. IRF4 levels however were significantly different in both experimental settings on day 10 (**Figure 10D**). IL-38 treated cells showed a 63.4-fold increase in IRF4 expression on day 4, before reducing to 60.2 on day 7 and 55.4 on day 10. B cells from the control group showed higher IRF4 expression on day 4 (not significant), which then decreased more strongly reaching a 22.9-fold change on day 10 ($p = 0.0397$).

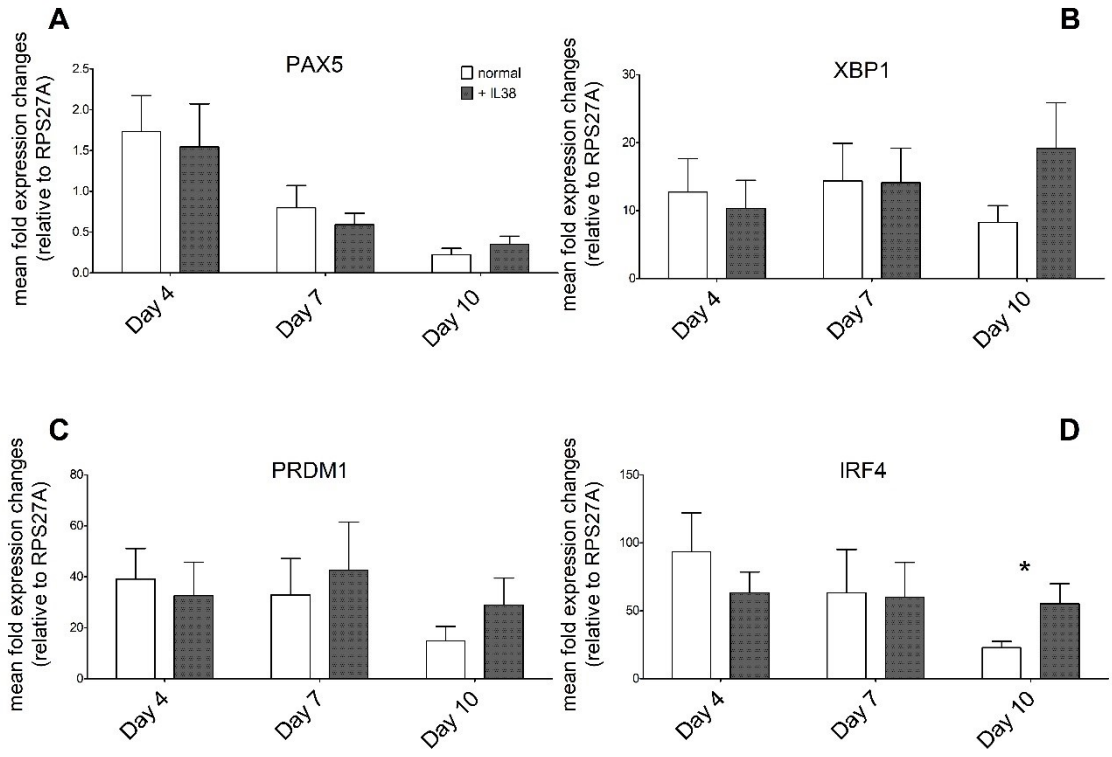


Figure 10: Impact of IL-38 on B cell specific gene expression
B cells from peripheral human blood were cultured in a three-step culture system as indicated above. The + IL-38 group received rhIL-38 at 130 ng/ml on day 0 and 7 and 60 ng/ml daily between day 3 and 6. At each culture step, cells were harvested and washed. RNA expression levels were determined by Real time qPCR. Data are mean expression levels with SEM relative to RPS27A. n = 6. *p < 0.05.

5.2.6 Additional IL-21 enhances B cell differentiation

So far, we had observed that IL-38 reduces the percentage of CD38 expressing B cells during in vitro differentiation, without significant influence on the percentage of plasma cells generated. In five additional experiments, we decided to add IL-21 as further stimulant with the purpose of enhancing B cell differentiation, creating more plasma cells and to examine if this would unmask further differences, possibly created through exogenous IL-38. In addition to the regular cytokines, IL-21 was used at 50 ng/ml on days 0, 4 and 7 of cell culture.

As expected, IL-21 treated B cells differentiated more rapidly (**Figure 11**). In the beginning, confirming our previous findings, 66.70% of all peripheral human blood B cells were mature naïve cells, 22.30% were positive for CD27 and almost no CD38 expressing cells were present (**Figure 11A**). After stimulation with the regular cytokines and the addition of IL-21, mnBC decreased more strongly than described before to a mean of 9.12% of all cells on day 4, 5.30% on day 7 and

2.68% on day 10. Meanwhile the percentage of CD27+ cells increased higher than we had seen without adding IL-21. On day 4, 58.61% of all cells expressed CD27. On day 7 a mean 87.89% and on day 10 93,49% of cells in culture were CD27+ cells. Simultaneously, CD38 activation was also clearly increased by addition of IL-21. On day 4, a mean 22,73% of all cells were CD38+, 54,58% on day 7 and 71,80% on day 10. In comparison, only 28,63% of day 10 cells were CD38+ without the addition of IL-21 (see **Figure 6**). As a result of higher CD38 expression, also more plasmablasts and plasma cells were detectable. On day 10 a mean 62.54% of CD27+ cells were considered plasmablasts and 13.60% plasma cells (**Figure 11B**). Hence, the addition of IL-21 to our differentiation protocol almost doubled the percentage of plasma cells generated until day 10. In contrast to the experiments without IL-21, the CD27+ CD38- cell population could not be further divided in two groups of MHC-II+ memory B cells and MHC-II- Pre-PB. Therefore, for analysis the CD27+ group was only further separated into CD38+/- cells.

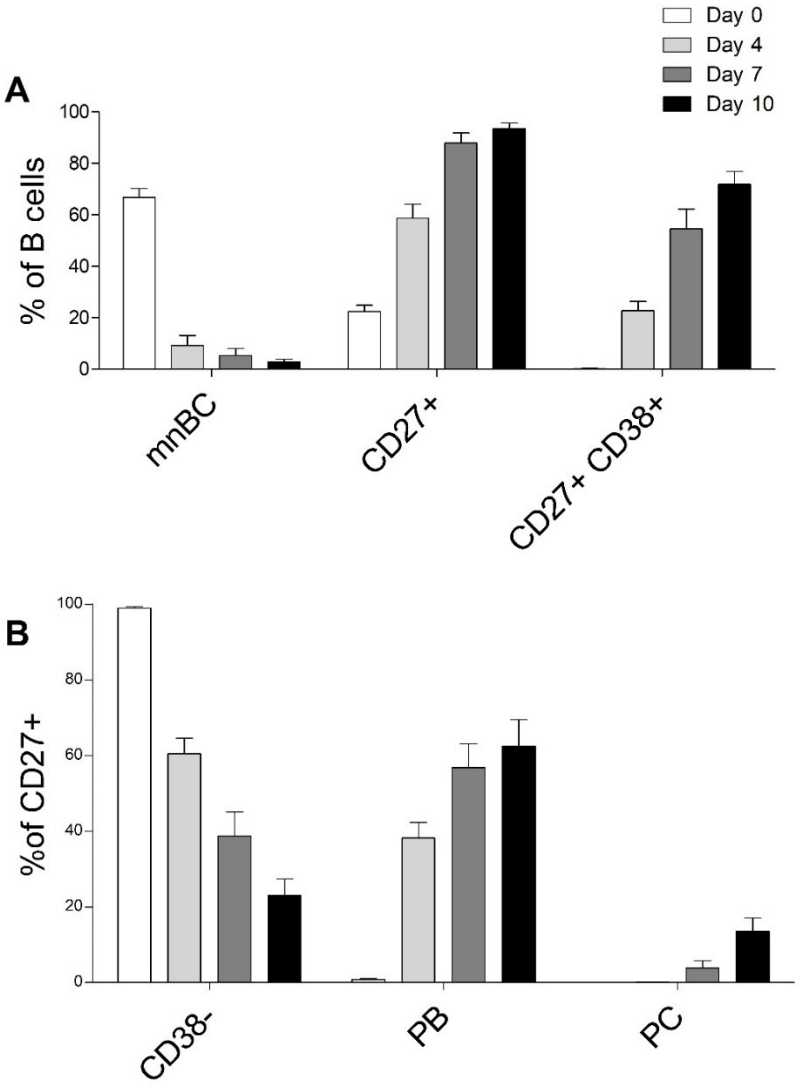


Figure 11: B cell differentiation under the impact of IL-21
B cells from peripheral human blood were cultured in a three-step culture system. The cells were initially stimulated with CpG ODN (20 µg/ml), sCD40-L (100 ng/ml), IL-2 (40 U/ml), IL-10 (100 ng/ml) and IL-15 (20 ng/ml) for 4 days. On day 4, the cells were washed and cultured with IL-2 (40 U/ml), IL-6 (100 ng/ml), IL-10 (100 ng/ml) and IL-15 (20 ng/ml) until day 7. After washing, the cells were cultured for three additional days with IL-6 (100 ng/ml), IL-15 (20 ng/ml) and IFN-α (100 U/ml). IL-21 was applied at 50 ng/ml on days 0, 4 and 7. At each culture step, the cells were harvested and analyzed by flow cytometry, using fluorochrome conjugated mABs to determine cell subsets. Data are mean percentages with SEM of all B cells (**A**) and of CD27+ cells (**B**). n = 5. mnBC: mature naïve B cells, PB: plasmablasts, PC: plasma cells

5.2.7 IL-21 reduces the effect of IL-38 on CD38 expression

After adding IL-21 to our regular B cell differentiation assay we again compared IL-38 treated B cells with a control group. Phenotypic changes were assessed using flow cytometry on days 0, 4, 7 and 10 of B cell differentiation. On day 4 of

the culture process, we did not find any differences in cell populations between control and treatment group (data not shown). This result is congruent with our previous findings from the experiments without additional IL-21. When analyzing percentages of all B cells on day 7, we saw the same tendencies we had observed before (**Figure 12A, B**). Cells treated with IL-38 showed less CD38 activation compared to the control cells. However, we could not find statistically significant differences between both groups. 33.25% of all cells in the control group were CD27+ CD38- cells compared to 37.42% of IL-38 treated cells (not significant). Vice versa, CD27+ CD38+ cells comprised for 55.58% in the control group and 52.43% in the treatment group (not significant). Plasma cells made up for a mean of 3.44% and 2.14% in control and treatment group ($p = 0.0625$). Looking at the subgroup of D7 CD27+ cells, we found a difference in the share of plasma cells, as IL-38 treatment reduced the percentage of plasma cells from 3.90% to 2.46%, however closedly failing statistical significance ($p = 0.0625$). On day 10 of in vitro differentiation, almost all remaining cells were expressing CD27, without differences between control and treatment group (**Figure 12C**). Of these CD27+ cells, 23.03% of control group cells were not expressing CD38. This was significantly lower compared to 25.56% in the IL-38 treated cells ($p = 0.0201$). Therefore, the proportion of CD38+ cells again tended to be higher in the cells not receiving exogenous IL-38 with 76.68% compared to 74.24% ($p = 0.0515$). The difference observed in D7 plasma cells was not statistically significant on day 10, as 13.60% in the control and 12.57% in the +IL-38 group of D10 CD27+ cells were defined plasma cells (**Figure 12D**).

In summary, with the addition of IL-21, we generally detected more CD27 and CD38 expression on the B cells and in the end created more plasma cells. The tendencies of less CD38 expression in IL-38 treated cells were reproduced but blunted compared to the assay without IL-21 stimulation (**Figure 12E).**

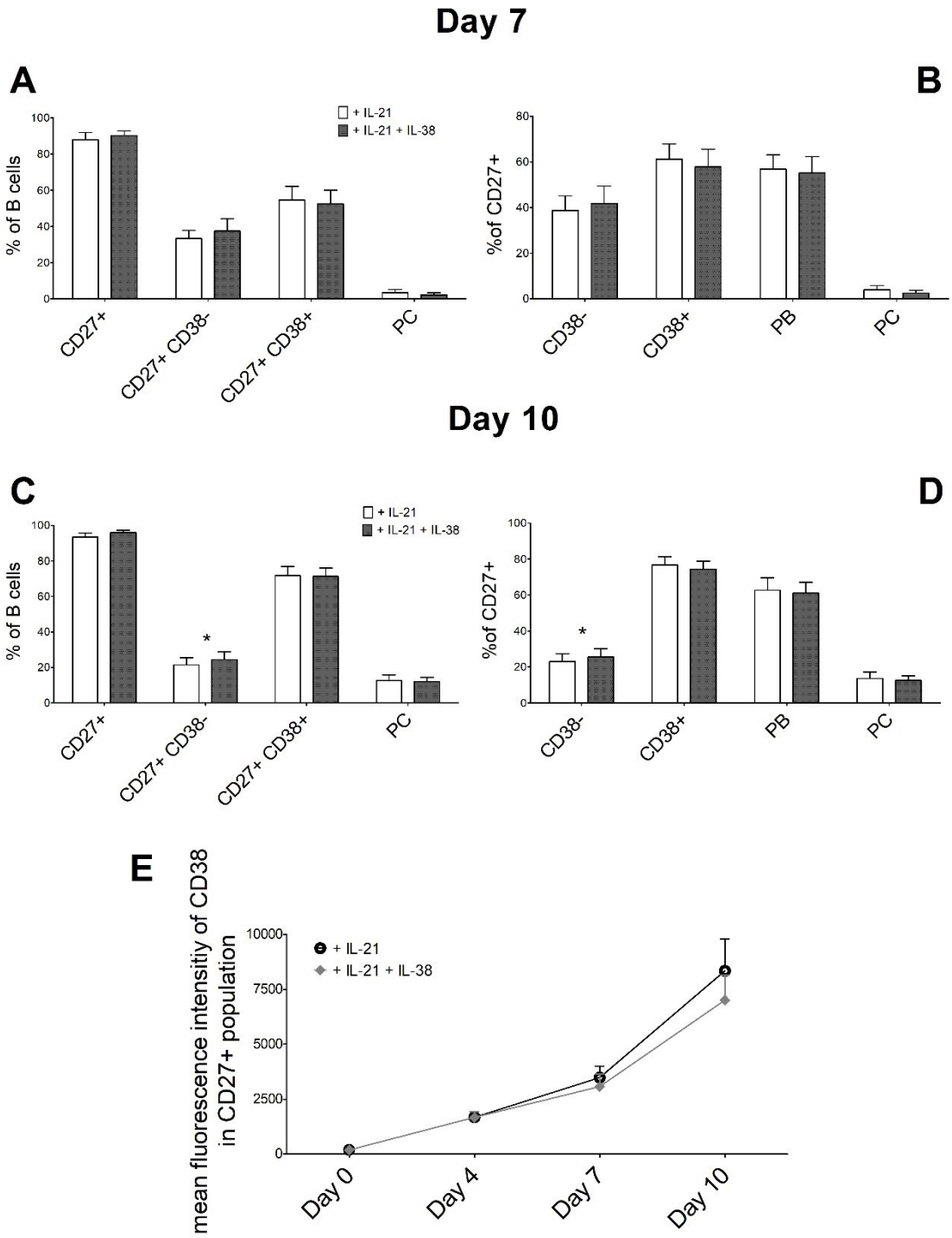


Figure 12: B cell differentiation under the impact of IL-38 in combination with IL-21

B cells from peripheral human blood were cultured in a three-step culture system as indicated above. In addition to the regular cytokines, IL-21 was applied at 50 ng/ml on days 0, 4, and 7. The + IL-38 group received rhIL-38 at 130 ng/ml on day 0 and 7 and 65 ng/ml daily between day 3 and 6. At each culture step, the cells were harvested and analyzed by flow cytometry, using fluorochrome conjugated mABs to determine cells subsets. Data are mean percentages with SEM of all B cells (**A, C**) and of CD27+ cells (**B, D**). n = 5. *p < 0,05. mnBC: mature naïve B cells, MBC: memory B cells, PB: plasmablasts, PC: plasma cells

5.2.8 IL-21 blocks the effects of exogenous IL-38 on immunoglobulin production

As we had seen the effect of IL-38 on differentiating B cells being decreased by adding IL-21 to our in vitro differentiation protocol, we also wanted to test changes in immunoglobulin production. Hence, we again collected supernatant from cell culture on days 4, 7 and 10 and determined Ig concentrations using Human total Ready-SET-Go!® ELISA kits according to the providers manual. As before, supernatants were used at optimal dilutions in PBS, 1:10 for IgM, 1:25 for IgA and 1:75 for IgG. In contrast to the experiments performed without IL-21, we did not find any statistically relevant differences between the control group and IL-38 treated cells, when adding IL-21 to the cell culture.

Total IgM concentrations were lower than we had seen before, with 4618 ng/ml in the control group on day 4, 23057 ng/ml on day 7 and 7319 ng/ml on day 10 (**Figure 13A**). Supernatants from the treatment group reached lower concentrations, without statistical significance becoming evident. The production of IgM per cell and day was also smaller compared to our previous experiments. The difference in D10 IgM production we had seen without IL-21 could not be replicated in the presence of IL-21. IgG concentrations in the supernatant and IgG production per cell and day were clearly increased by the addition of IL-21 as stimulant (**Figure 13C, D**). On day 4, we detected a mean of 1250 ng/ml in supernatants of the control group, 10098 ng/ml on day 7 and 7449 ng/ml on day 10. Again, no significant differences were detectable in the +IL-38 group. Only on day 7 IgG concentrations in supernatants of IL-38 treated cells seemed decreased with 6972 ng/ml ($p = 0.0518$). However, analyzing IgG production per cell and day, no relevant differences could be confirmed. As our previous experiments indicated, IgG production per cell and day increased constantly from day 4 (1.05 pg/cell/day) until day 7 (24.52 pg/cell/day) and day 10 (49.32 pg/cell/day). The measured production of IgA was also more than doubled by the addition of IL-21 to the in vitro differentiation assay (**Figure 13E, F**). Total IgA concentrations in the control group reached 1827 ng/ml on day 4, 9509 ng/ml on day 7 and 4129 ng/ml on day 10. IgA production measured per living cell and days in culture were 1.62, 24.92 and 30.14 pg/cell/day on days 4, 7 and 10. Neither the total concentration of IgA in supernatants, nor the calculated

production per cell and day, depicted significant differences between control and IL-38 treatment group.

All in all, the addition of IL-21 to our in vitro assay limited or complete blocked all changes IL-38 conferred in B cell differentiation we had observed before. It seems that in the presence of IL-21, IL-38 does not have a significant influence on the immunoglobulin production of B cells. Interestingly, IL-21 enhances the production of IgG and IgA, while decreasing IgM secretion.

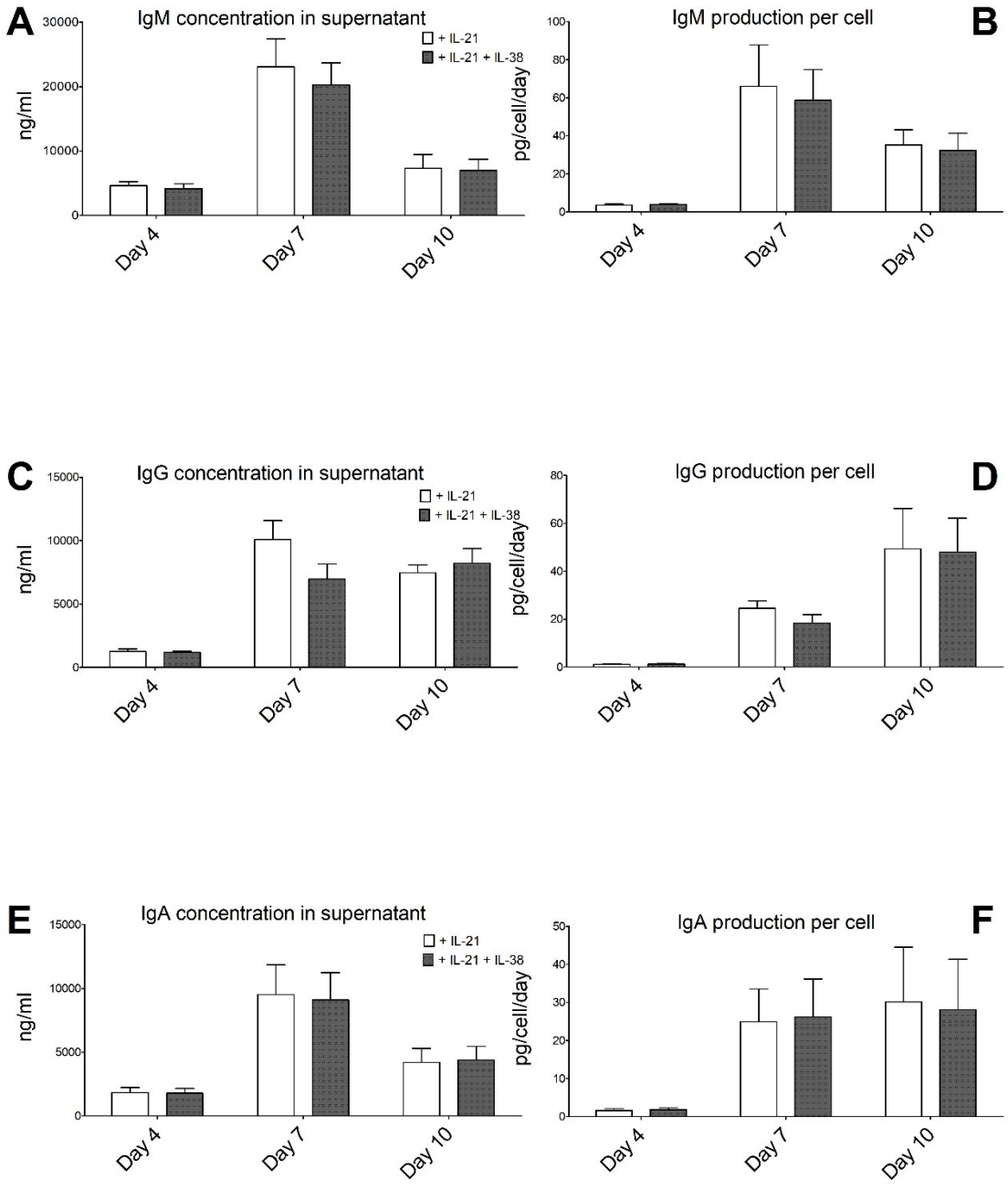


Figure 13: Immunoglobulin production in the presence of IL-21
B cells from peripheral human blood were cultured in a three-step culture system as indicated above. The + IL-38 group received rhIL-38 at 130 ng/ml on day 0 and 7 and 65 ng/ml daily between day 3 and 6. At each culture step, supernatant was obtained and analyzed by ELISA using Human total Ready-SET-Go!® ELISA kits for IgA, IgG and IgM. Data are mean immunoglobulin concentrations in cell culture supernatants (**A, C, E**) and immunoglobulin production per cell and day (**B, D, F**) with SEM. n = 5.

5.2.9 B cell specific genes are not affected by IL-38 in the presence of IL-21

Next, we wanted to test how the addition of IL-21 to the B cell differentiation protocol affected gene expression levels of B cell specific genes and IL-38-receptors. Our flow cytometric data showed B cell treatment with additional IL-21 to be a potent enhancer of B cell differentiation creating more plasma cells at the end of the in vitro differentiation protocol. Therefore, we also expected this to be evident in expression levels of genes involved in B cell differentiation. For statistical purposes, due to the low number of replicates, we predominantly could not test for normal distribution and therefore only assumed normal distribution in these cases. For all inspected genes we did not find a significant difference created through treatment with exogenous IL-38. PAX5 expression levels decreased more clearly and earlier than observed without IL-21 as stimulant (**Figure 14A**). After ten days the mean fold changes were 0.124 and 0.116 in control and treatment group. Fold changes of XBP1 expression were increased compared to our previous differentiation assay with 27.72 on day 4, 21.12 on day 7 and 20.51 on day 10 in control group cells (**Figure 14B**). The development of PRDM1 expression was comparable to our previous experiments as fold changes decreased from 40.66 to 19.47 between days 4 and 10 (**Figure 14C**). Interestingly, IRF4 expression levels were decreased by the additional use of IL-21 as stimulant with fold changes of 30.96 on day 4, 22.20 on day 7 and 20.93 on day 10. In our previous experiments, IL-38 treatment significantly increased IRF4 expression on day 10 of the differentiation process. This change was not evident when B cells were also stimulated with IL-21 (**Figure 14D**).

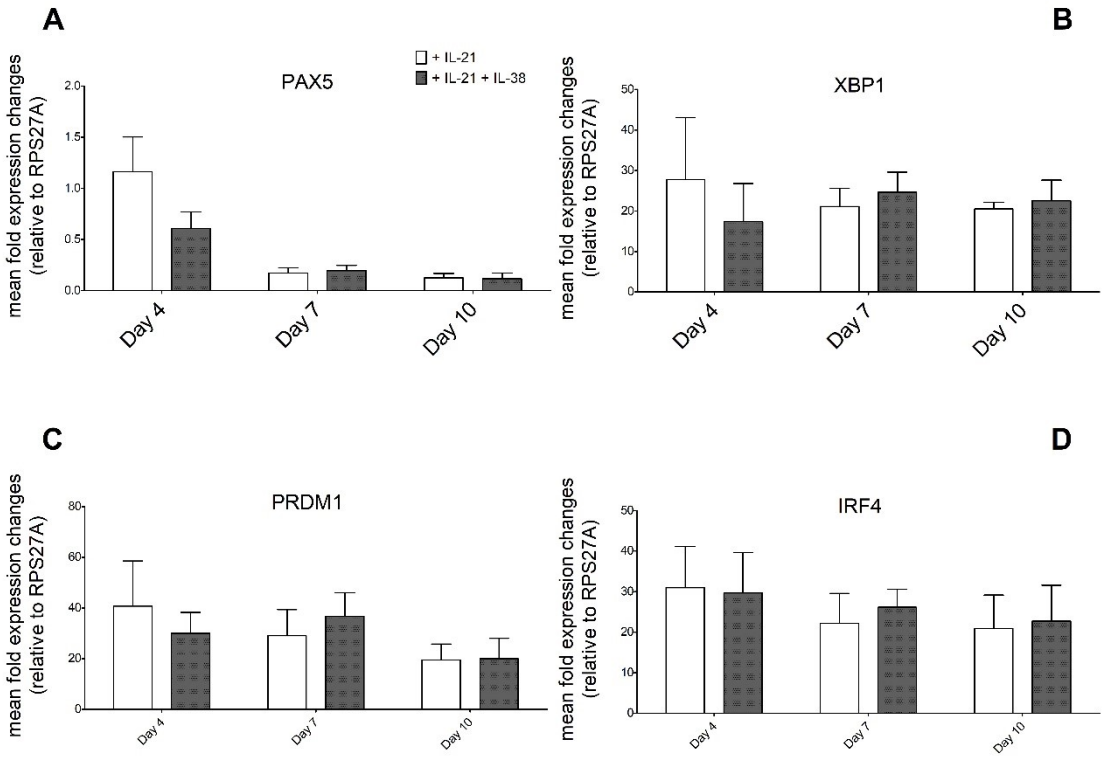


Figure 14: B cell specific gene expression in IL-21 B cell differentiation protocol
B cells from peripheral human blood were cultured in a three-step culture system as indicated above. The + IL-38 group received rhIL-38 at 130 ng/ml on day 0 and 7 and 65 ng/ml daily between day 3 and 6. At each culture step, cells were harvested and washed. RNA expression levels were determined by Real time qPCR. Data are mean fold expression changes with SEM relative to RPS27A. n = 4.

We also investigated how additional IL-21 treatment affected gene expression levels of IL-38 receptors during B cell differentiation. In the regular protocol of B cell differentiation without IL-21, gene expression levels were consistently determinable for IL-36R, while less reliably for IL-1R1 and IL-1RAPL1 prohibiting profound statistical analysis. With IL-21 as additional stimulant IL-1RAPL1 and IL-36R were detected in all and IL-1R1 in four out of five donors. Concordant with our previous findings, exogenous IL-38 did not have a significant influence on IL-38 receptor expression levels. IL-21 stimulation itself altered expression of IL-1R1 mRNA, as upregulation between day 0 and day 4 was clearly reduced compared to those we had observed without IL-21 treatment (**Figure 15A**). Development of IL-36R expression over the differentiation process was comparable to our previous data as fold expression changes in the control group were 1.37 on day 4, 0.85 on day 7 and 0.58 on day 10 (**Figure 15B**). Analyzing IL-1RAPL1 expression changes, we observed different tendencies for control and

treatment group (**Figure 15C**). Congruent with our previous findings concerning IL-1RAPL1, expression levels in the control group decreased in the first four days of in vitro differentiation by a fold change of 0.15. In contrast to the experiments without IL-21, IL-1RAPL1 expression then remained on this level with fold changes of 0.19 and 0.15 on days 7 and 10. However, the treatment group showed the same tendency of increasing expression towards the end of differentiation as fold changes reached 0.43 and 1.05 on days 7 and 10. The differences between control cells and IL-38 treated cells were not statistically significant.

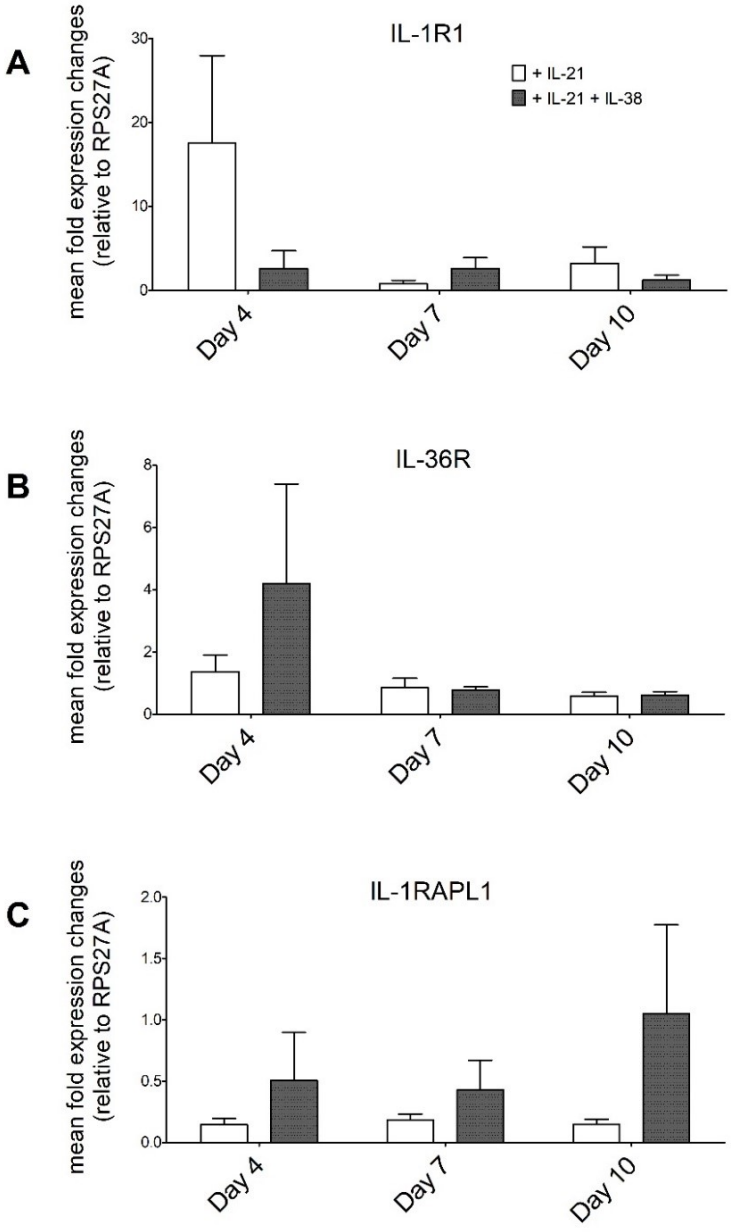


Figure 15: IL-38 receptor gene expression in IL-21 B cell differentiation protocol
B cells from peripheral human blood were cultured in a three-step culture system as indicated above. The + IL-38 group received rhIL-38 at 130 ng/ml on day 0 and 7 and 65 ng/ml daily between day 3 and 6. At each culture step, cells were harvested and washed. RNA expression levels were determined by Real time qPCR. Data are mean fold expression changes with SEM relative to RPS27A. n = 4 (A). n = 5 (B, C).

5.3 Impact of IL-38 on B cell proliferation and cell death

5.3.1 IL-38 reduces cell survival of differentiating B cells

In our experiments testing differences in B cell differentiation, we observed that, in the group of IL-38 treated cells, the number of living cells tended to be lower compared to the control group. However, in these experiments, cells considered to be alive were only defined by their physical properties in the first gating step of flow cytometric analysis.

The cell concentration in culture was calculated by using a counting standard (counting beads) and the volume of cell culture material harvested for flow cytometry. In the protocol of naïve and memory B cells differentiating to plasma cells without IL-21 stimulation, we found a tendency for lower cell numbers under the influence of IL-38 treatment (**Figure 16A**). This tendency was small on day 4 of the differentiation process as a mean of 305000 cells/ml were defined alive in the control group and 296000 cells/ml in the treatment group. On day 7 the cell count was higher in the control group compared to the IL-38 group in five out of six experiments. However, taking all six experiments into account, the difference closely missed statistical significance ($p = 0.0625$, Wilcoxon matched-pairs signed rank test). After day 7, the number of cells in culture decreased in both experimental groups, while the tendency of reduced living cells in the IL-38 group remained. At the end of the in vitro differentiation protocol, a mean of 63000 cells/ml were present in the control and 51000 cells/ml in the +IL-38 group ($p = 0.0715$). The experiments performed with additional IL-21 revealed similar tendencies (**Figure 16B**). Generally, IL-21 did not significantly increase the number of cells in culture even though it proved itself as potent enhancer of B cell differentiation. The tendency of lower cell concentrations in the IL-38 treatment group remained, however not statistically significant. On day 4, more living cells were present in the control group in four out of five experiments. The mean cell concentration was 337000 per ml in the control group and 266000 cells/ml in IL-

38 treatment group (p = 0.0743). After that, the number of cells decreased to 150000 cells/ml on day 7 and 77000 cells/ml on day 10 in the control group.

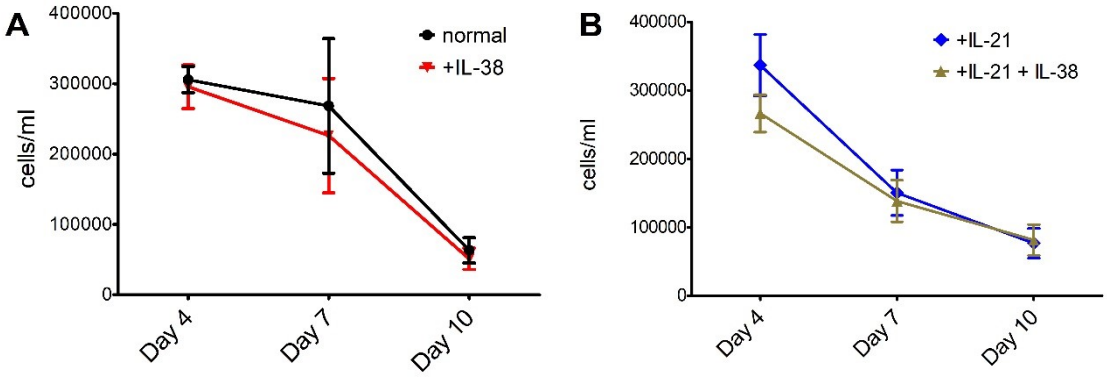


Figure 16: IL-38 treatment decreases B cell viability during in vitro differentiation
B cells from peripheral human blood were cultured in a three-step culture system as described above. The cells were initially stimulated with CpG ODN (20 µg/ml), sCD40-L (100 ng/ml), IL-2 (40 U/ml), IL-10 (100 ng/ml) and IL-15 (20 ng/ml) for 4 days. On day 4, the cells were washed and cultured with IL-2 (40 U/ml), IL-6 (100 ng/ml), IL-10 (100 ng/ml) and IL-15 (20 ng/ml) until day 7. After washing, the cells were cultured for three additional days with IL-6 (100 ng/ml), IL-15 (20 ng/ml) and IFN-α (100 U/ml). Each step was performed with (**B**, n = 5) or without (**A**, n = 6) addition of 50 ng/ml IL-21. The + IL-38 group received rhIL-38 at 130 ng/ml on day 0 and 7 and 65 ng/ml daily between day 3 and 6. At each culture step, the cells were harvested and analyzed by flow cytometry. Living cells were determined by their FSC and SSC characteristics (size and granularity) via flow cytometry. Data are mean concentrations of cells in culture with SEM. *p < 0.05.

5.3.2 IL-38 promotes cell death during B cell differentiation

In order to investigate the influence of IL-38 on B cell proliferation and cell death more closely, we constructed an experiment testing proliferation and cell death simultaneously. To that end, we added a fluorescent proliferation dye to our in vitro differentiation protocol. After isolating B cells from peripheral human blood of four different donors, B cells were incubated with Cell proliferation dye eFluor 670. Afterwards, the B cells were subjected to our differentiation protocol. The cells were separated in four different groups, for which no additional cytokines or IL-38 +/- IL-21 were added. On days 4 and 7, the cells were harvested and analyzed by flow cytometry. Cell proliferation was determined by fluorescence intensity of the proliferation dye, with reduced intensity indicating higher proliferation. We further stained for CD27 expression with anti CD27 mABs and combined this with an Annexin-V/propidium iodide staining to differentiate apoptotic, necrotic, and viable cells. Hence, we first gated the cells by their physical properties to remove cell debris and then separated them into CD27+/-

cells as well as alive, apoptotic, or necrotic cells, depending on Annexin-V and/or propidium iodide positivity. Annexin-V binds to phosphatidylserine which is present on the outer surface of apoptotic cells and therefore marks cells undergoing apoptosis. Necrotic cells lose their membrane stability and allow Annexin-V and propidium iodide to enter the cell. Therefore, apoptotic cells can be distinguished from necrotic ones as they are only positive for Annexin-V staining, necrotic cells being double positive and living cells being double negative.

First, we could confirm our findings from the previous experiments regarding CD27 expression, as we observed that exogenous IL-38 did not affect the proportion of CD27+ cells on days 4 and 7 of B cell differentiation, independent of IL-21 stimulation. However, the addition of IL-21 again clearly increased the percentage of CD27+ cells (data not shown). Analyzing day 4 cells (**Figure 17A**), we first explored the percentage of living cells in culture. In the group of cells not receiving any additional cytokine, 93.51% of the gated cells were alive compared to 92.80% in the IL-38 treated group ($p = 0.1303$). This proved our previous method of defining cell viability by physical properties to be sufficient. In both groups, around 4% of all cells were apoptotic. The percentage of necrotic cells was slightly but significantly increased under the influence of IL-38 with 2.20% in the untreated group and 2.59% in the IL-38 treated cells ($p = 0.0275$). In the presence of IL-21, this effect was even stronger. Here, 2.57% of all cells were defined as necrotic in the IL-21-treated control group, compared to 3.44% in the IL-21 and IL-38 treated group ($p = 0.0115$). Furthermore, when IL-21 was added, IL-38 also raised the percentage of apoptotic cells in culture from 10.56% to 12.16% ($p = 0.0097$). Consequently, the mean percentage of living cells was decreased from 86.69% of IL-21 treated cells to 84.07% when treated additionally with IL-38 ($p = 0.0058$). Interestingly, necrosis was more prominent in the group of CD27+ cells, while apoptosis predominantly occurred in the CD27- population (**Figure 17B, C**). We observed that IL-38 treatment reduced B cell survival in CD27+ cells on day 4. This observation became statistically significant only when IL-21 was also added. The percentage of living CD27+ cells was decreased from 91.10% to 88.60% ($p = 0.0166$) and resulted from an increase in necrotic cells from a mean 7.39% to 9.99% of CD27+ cells ($p = 0.0199$). Looking closer at the

D4 CD27- cell population, we did not find a relevant effect of IL-38 on cell death, neither in the regular differentiation protocol, nor in the presence of IL-21 (**Figure 17C**). As IL-21 is known to exert an apoptotic influence on CD27- mature naïve B cells, the proportion of apoptotic cells was clearly increased compared to CD27 expressing cells.

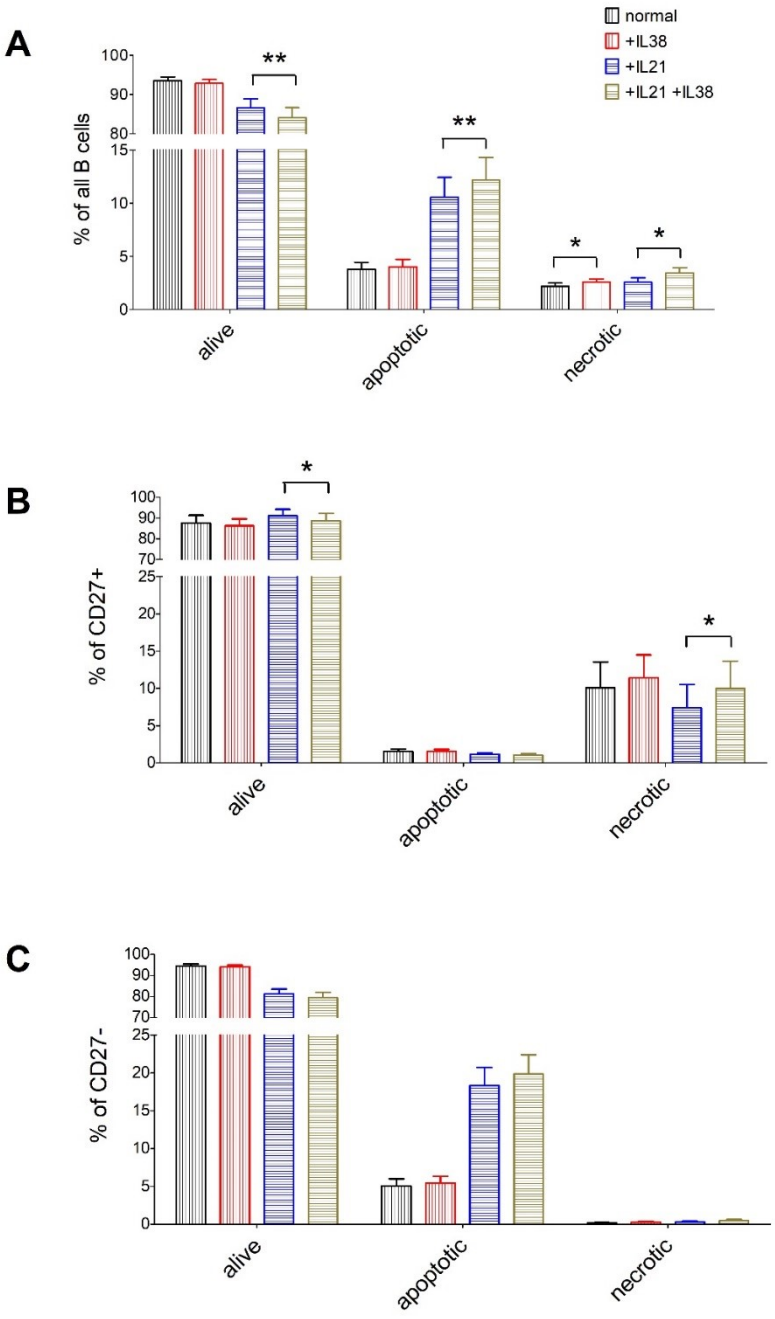


Figure 17: IL-38 increases apoptosis and necrosis of B cells on day 4 of in vitro differentiation
B cells were obtained from peripheral human blood and cultured in a two-step culture system for seven days. Four conditions of B cell differentiation were used. One half of cells received additional IL-21 at 50 ng/ml on days 0 and 4. Both groups were separated

into control cells and IL-38 treated cells receiving rhIL-38 at 130 ng/ml on day 0 and 65 ng/ml daily between day 3 and 7. Cell death was determined by Annexin-V/Propidium Iodide staining after four days in culture. Data are mean percentages with SEM related to all cells (**A**), CD27+ cells (**B**) or CD27- cells (**C**). n = 4. *p < 0.05. **p < 0.01.

After seven days of in vitro B cell differentiation, the percentage of all living cells in culture had decreased compared to day 4, reaching around 80% in all four culture conditions (**Figure 18A**). The proportion of apoptotic cells increased during that time span reaching between 15-17% in all four experimental settings. The effect of IL-38 increasing the percentage of necrotic cells was also evident on day 7. When B cells were stimulated with the regular cytokines, IL-38 significantly raised the proportion of necrotic B cells from 3.03% to 4.66% of all D7 cells ($p = 0.0047$). Under the additional influence of IL-21, IL-38 treated cells also showed increased necrosis with 2.57% compared to 1.38% without IL-38 ($p = 0.108$). As we had already observed on day 4, necrotic cells were mainly present in the group of CD27+ cells and were again increased by IL-38 treatment on day 7 (**Figure 18B**). Under IL-38 influence the proportions of necrotic CD27+ cells were raised from 4.95% to 7.29% ($p = 0.0085$) without IL-21 and from 1.35% to 2.84% ($p = 0.0122$) with the additional stimulation by IL-21. Consequently, proportions of alive CD27+ cells were also significantly reduced in IL-38 treatment groups. In contrast to CD27+ cells, the CD27- cell population showed high apoptosis after seven days in culture, particularly in IL-21 treated cells (**Figure 18C**). As we had observed on day 4, IL-38 did not greatly influence the CD27- cells. However, IL-38 altered necrosis in D7 CD27- cells not being stimulated with IL-21, as the percentage of necrotic B cells was increased from 1.49% to 2.63% ($p = 0.0437$).

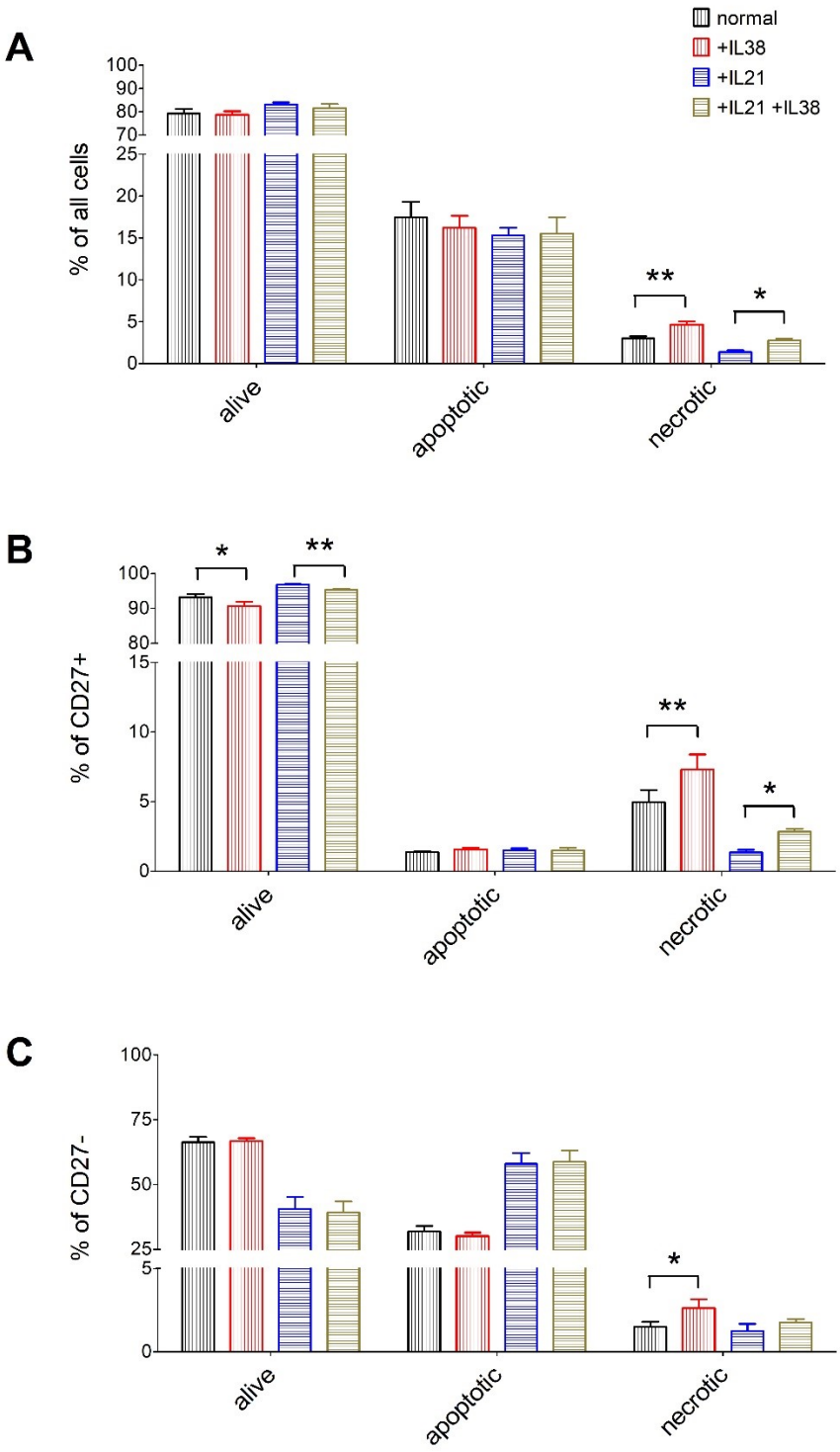


Figure 18: IL-38 increases apoptosis and necrosis of B cells on day 7 of in vitro differentiation

B cells were obtained from peripheral human blood and cultured in a two-step culture system for seven days. Four conditions of B cell differentiation were used. One half of cells received additional IL-21 at 50 ng/ml on days 0 and 4. Both groups were separated into control cells and IL-38 treated cells receiving rhIL-38 at 130 ng/ml on day 0 and 65 ng/ml daily between day 3 and 7. Cell death was determined by Annexin-V/Propidium Iodide staining after four days in culture. Data are mean percentages with

SEM related to all cells (**A**), CD27+ cells (**B**) or CD27- cells (**C**). n = 4. *p < 0.05. **p < 0.01.

5.3.3 B cell proliferation is reduced by exogenous IL-38

In the experimental setting testing cell death of differentiating B cells, we also added a fluorescent proliferation dye to investigate differences in B cell proliferation. As marker for proliferation, we analyzed the mean fluorescence intensity in flow cytometric analysis. Lower intensity indicates higher proliferation. In general, as expected CD27+ cells had proliferated more than B cells who have not acquired CD27+ phenotype. Also, cells being stimulated with IL-21 showed increased proliferation compared to the cells not receiving IL-21 in all tested cell subsets (**Figure 19**). On day 4, additional IL-38 significantly decreased proliferation of all IL-21 treated B cells by around 10% (p = 0.0462) and of CD27+ cells by around 12% (p = 0.0386). In the group of cells not receiving IL-21, additional IL-38 did not have a significant influence on B cell proliferation. On day 7, no differences in proliferation of CD27+ cells were evident. However, CD27- cells showed less proliferation at this time point, when treated with IL-38 only (p = 0.0366).

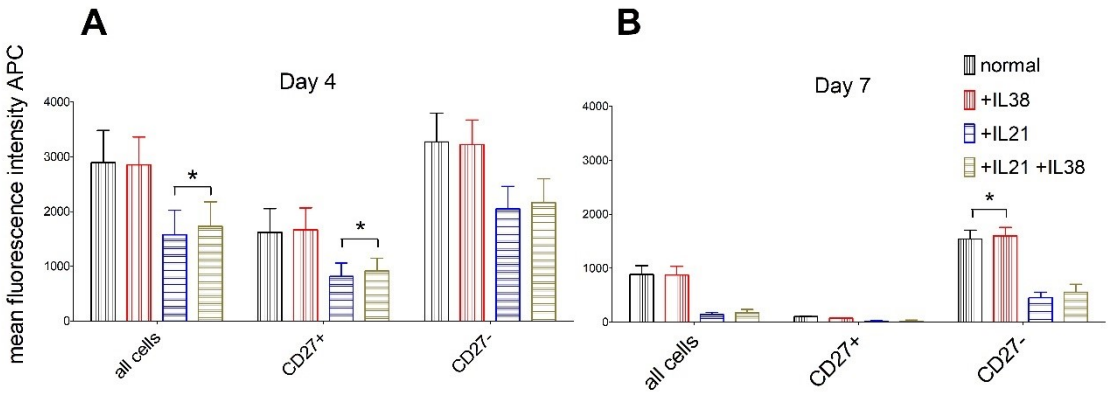


Figure 19: Analysis of B cell proliferation under the impact of exogenous IL-38
B cells were obtained from peripheral human blood and cultured in a two-step culture system for seven days. Four conditions of B cell differentiation were used. One half of cells received additional IL-21 at 50 ng/ml on days 0 and 4. Both groups were separated into control cells and IL-38 treated cells receiving rhIL-38 at 130 ng/ml on day 0 and 65 ng/ml daily between day 3 and 7. Cell proliferation was assessed via proliferation dye eFluor 670. Data are mean fluorescence intensities with SEM. n = 4. *p < 0.05.

5.4 Impact of IL-38 knockdown on B cell differentiation

So far, we focused on the influence of exogenous IL-38 on B cells differentiating into plasma cells. Next, we were interested if we could further detect an intrinsic function of IL-38 as B cells had been shown to produce IL-38 themselves.

5.4.1 IL-38 gene expression is evident in differentiating B cells

At first, we investigated if we could detect IL-38 mRNA in non-stimulated B cells from peripheral human blood and if its expression changed during the process of B cell differentiation. Therefore, we analyzed cell samples obtained from the six experiments of our regular B cell differentiation assay and the five experiments performed with additional IL-21 and examined IL-38 gene expression levels via qPCR. Indeed, IL-38 was present in day 0 B cells, mostly being mature naive B cells, in all tested donors (**Figure 20**). During the process of B cell differentiation with and without IL-21, increased IL-38 gene expression levels were found. It seemed, IL-38 expression increased between day 0 and day 4, before constantly decreasing again towards day 10. This trend was not replicable in all donors and varied widely between single experiments. Statistically significant outliers within these experiments were identified by Grubbs´ test and excluded from further analysis. Therefore, we only provide a descriptive analysis of IL-38 gene expression during B cell differentiation.

All in all, we did not observe significant differences in IL-38 expression between control group and IL-38 treated cells and due to the large variety of IL-38 expression between single samples cannot postulate a clear tendency of expression development during the process of B cell differentiation.

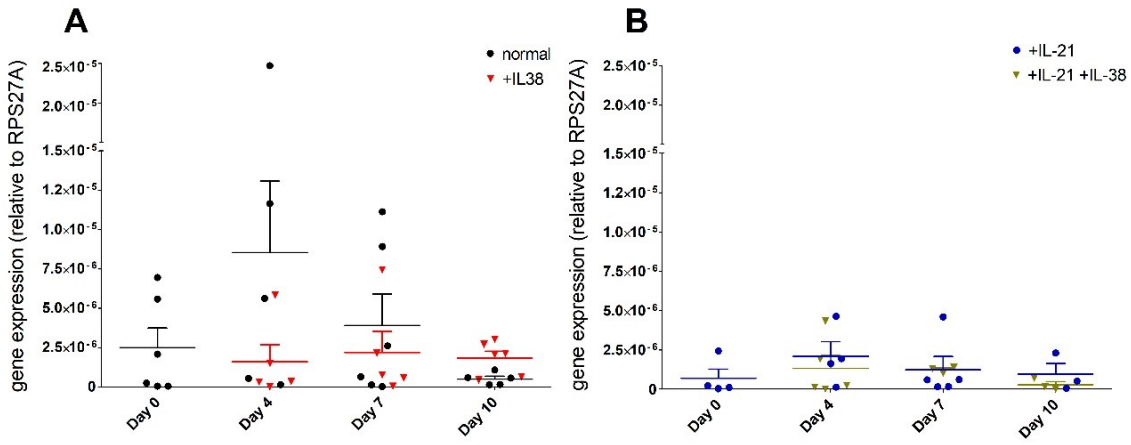


Figure 20: IL-38 gene expression in differentiating B cells
B cells from peripheral human blood were cultured in a three-step culture system as indicated above. The + IL-38 group received rhIL-38 at 130 ng/ml on day 0 and 7 and 65 ng/ml daily between day 3 and 6. Each step was performed with (**B**, n = 5) or without (**B**, n = 6) addition of 50 ng/ml IL-21. RNA expression levels were determined by Real time qPCR. Data are mean IL-38 expression levels with SEM relative to RPS27A.

5.4.1 Establishing IL-38 knockdown B cells

Finally, after confirming IL-38 expression in B cells, we attempted depleting endogenous IL-38 before differentiating them into plasma cells to examine the internal function of IL-38 in B cells. To perform an IL-38-knockdown, we used Accell SMARTpool Human IL1F10 siRNA compared with a Non-Targeting siRNA Pool #1 as reagent for the control group. As FCS inhibits siRNA function, we used FCS-free Accell siRNA Delivery Media containing 100 U/ml penicillin, 100 mg/ml streptomycin, 50 µg/ml transferrin and bovine Insulin (1:2000). The Accell system has the advantage not to require a separate transfection reagent and to avoid stimulation of RNA-recognizing TLRs,[216] which are expressed by B cells. After B cell isolation, the cells were incubated with IL-38 siRNA or non-targeting control at a concentration of 1 µM and subjected to a modified B cell differentiation protocol. Cells were stimulated according to our B cell differentiation assay with the application of 20 µl FCS to each well after 24 h of incubation. The siRNA treatment was not repeated after the initial incubation. From day 4 on, regular IMDM-medium containing FCS was used. At each culture step, cells were harvested for flow cytometry and gene expression was measured via qPCR. Also, supernatant from cell culture was taken to analyse immunoglobulin production by ELISA.

Four experiments were performed using B cells from different blood donors. First, we had to determine if the IL-38 knockdown was sufficient. Therefore, we analyzed IL-38 mRNA expression levels via qPCR. In our previous experiments we had observed that IL-38 levels varied widely between different donors but tended to be increased on day 4 of the differentiation process before decreasing towards day 10. We were most interested in the expression levels after four days of cell stimulation to compare expression levels between the control and knockdown group. In one of the four donors we detected higher IL-38 expression in the knockdown group compared to the control. Consequently, this donor was declared a failed knockdown approach and excluded from all further analysis of the IL-38 knockdown experiments. In the other three donors, IL-38 expression was lower in the cells treated with IL-38 siRNA compared to the control group treated with non-targeting siRNA. The mean IL-38 gene expression in knockdown cells was significantly reduced to 44.23% of their respective control ($p = 0.0252$, one-tailed t-test).

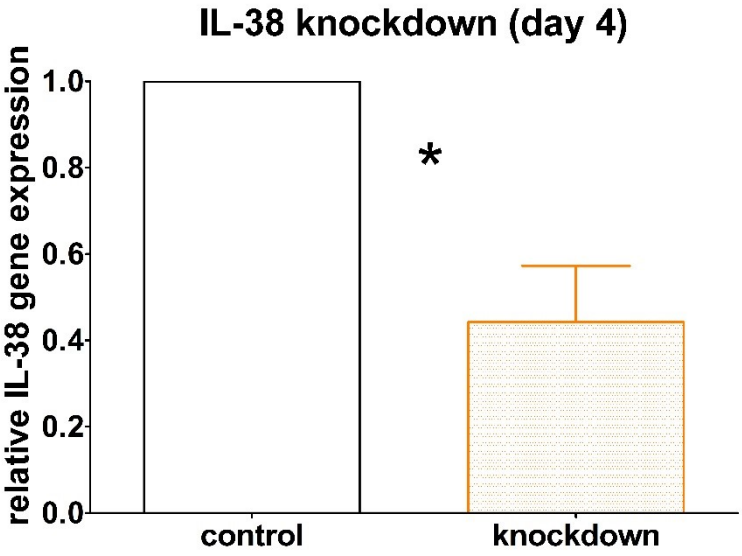


Figure 21: Successful IL-38 knockdown at day 4 during B cell differentiation
B cells were obtained from peripheral human blood via negative selection. Knockdown cells were initially incubated with Accell SMARTpool Human IL1F10 siRNA at 1 µM, the control group was treated with Non-Targeting siRNA Pool #1 at 1 µM. All cells were stimulated with CpG ODN (20 µg/ml), sCD40-L (100 ng/ml), IL-2 (40 U/ml), IL-10 (100 ng/ml) and IL-15 (20 ng/ml) for 4 days. RNA expression levels were determined by Real time qPCR. Data are mean gene expression levels normalized to the control group and relative to RPS27A. n = 3. *p < 0.05.

On day 7 and 10 of the B cell differentiation process, there was no difference in IL-38 expression visible, which was foreseeable as we had not repeated the initial

siRNA treatment (data not shown). Due to the low number of replicates, we could not test for normal distribution. Therefore, for all statistical analysis concerning the knockdown experiments we only assumed normal distribution.

5.4.2 Differentiating IL-38 knockdown cells show tendencies of increased CD38 expression

We then analyzed the distribution of B cell subtypes during the differentiation process of naïve and memory B cells to plasma cells between knockdown and control group (**Figure 22** (**Figure 5**).). On day 4 of differentiation, no statistically significant changes brought about by reduced IL-38 expression were detectable on a phenotypical level. Confirming our previous results from the regular B cell differentiation assay (**Figure 6**), mature naïve B cells comprised for around 40% of all B cells in both experimental groups while CD27+ cells accounted for a mean of 39.41% in the control and 36.33% in the knockdown group. However, the proportion of CD38+ cells showed different results than observed before. All experiments treating B cells with exogenous rhIL-38 showed at least slightly reduced percentages of CD38+ cells. Even though not all these results were statistically relevant the tendency was constantly present. Now, the group of cells with reduced IL-38 gene expression revealed the opposite tendency presenting higher CD38 expression. In CD27+ cells, the mean percentages of CD38+ cells were 30.55% in the control and 37.91% in the knockdown group (**Figure 22B**). Due to the low number of replicates, no statistical significance was evident. However, we believe this tendency to be relevant, as two of the three experiments showed strong increases in CD38+ cells with mean changes of 7.67% and 20.14%. The mean fluorescence intensity of CD38 in flow cytometry supports these findings of increased CD38 expression (**Figure 22E**). On day 7 this tendency was not further visible. In the group of CD27+ cells, no differences between control and knockdown group occurred in any of the analyzed cell types (data not shown). However, looking at percentages of all B cells, changes in CD27+ cells were detectable (**Figure 22C)**. The control cells showed significantly higher percentages of CD27+ cells with a mean of 65.92% compared to 56.11% in knockdown cells ($p = 0.0019$). Consequently, the proportion of mature naïve cells was higher in the knockdown group with 36.61% compared to 28.01% of all B cells ($p = 0.0142$). On day 10 the tendency of increased CD27 expression in

the control group remained (**Figure 22D**). 60.05% of all B cells were expressing CD27 in the control group and 53.21% in the knockdown cells (not significant). Also, the share of CD27+ CD38+ cells was decreased in the knockdown cells, with 34.27% compared to 29.13% of all B cells ($p = 0.0092$). This difference in CD38+ cells was not significant analyzing percentages of CD27+ cells alone, with 55.92% in the control and 52.76% in the knockdown group (data not shown). The proportion of plasma cells was not affected by the IL-38 knockdown at any time point. On day 10, a mean 4.78% and 4.45% of CD27+ cells were plasma cells in control and knockdown group.

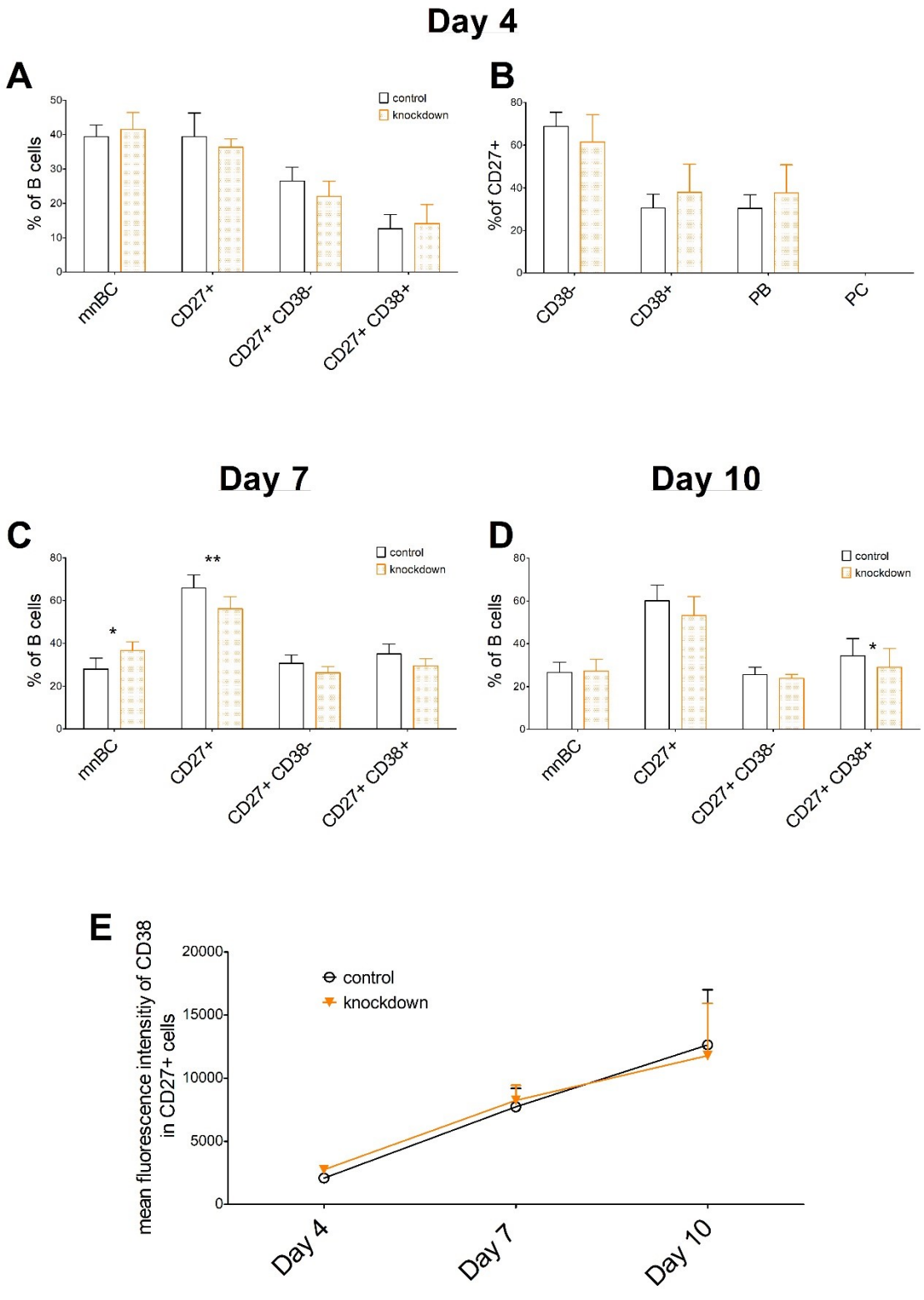


Figure 22: B cell subtypes during B cell differentiation of IL-38 knockdown cells
B cells from peripheral human blood were isolated and cultured in a three-step culture system for a total of ten days. Knockdown cells were initially incubated with Accell SMARTpool Human IL1F10 siRNA at 1 µM, the control group was treated with Non-Targeting siRNA Pool #1 at 1 µM. All cells were stimulated with CpG ODN (20 µg/ml), sCD40-L (100 ng/ml), IL-2 (40 U/ml), IL-10 (100 ng/ml) and IL-15 (20 ng/ml) for 4 days.

On day 4, the cells were washed and cultured with IL-2 (40 U/ml), IL-6 (100 ng/ml), IL-10 (100 ng/ml) and IL-15 (20 ng/ml) until day 7. After washing, the cells were cultured for three additional days with IL-6 (100 ng/ml), IL-15 (20 ng/ml) and IFN-α (100 U/ml). At each culture step, the cells were harvested and analyzed by flow cytometry, using fluorochrome conjugated mABs to determine cell subtypes. Data are mean percentages with SEM of all B cells (**A, C, D**) and of CD27+ cells (**B**) or mean fluorescence intensity of CD38 obtained from flow cytometry (**E**). n = 3. *p < 0.05. **p < 0.01. mnBC: mature naïve B cells, PB: plasmablasts, PC: plasma cells

We were also interested to investigate the influence of an IL-38 knockdown on the number of living cells in culture. Our proliferation and cell death assays had shown that exogenous IL-38 increased cell death of B cells (**see 5.3.2**). Now, with reduced IL-38 gene expression, we also expected the opposite effect. We analyzed the number of living cells in culture of the three knockdown experiments by flow cytometry using physical properties. In general, the overall cell count was in the same range as in our previous experiments, showing siRNA treatment itself and the initial 24 h without FCS in the culture medium did not relevantly affect proliferation and cell death (**Figure 23)**. On day 4, 415000 cells/ml were present in the control and 380000 cells/ml in the knockdown group (not significant). On day 7, the number of cells in the control group increased to 500000 cells/ml while the knockdown group remained at 380000 cells/ml. This difference was almost statistically significant ($p = 0.0701$) and did not confirm our expectations of increased cell counts in the knockdown group. At the end of the in vitro differentiation, 155000 cells/ml were alive in the control and 135000 in the knockdown cells (not significant). It seems as reduced levels of endogenous IL-38 are also associated with decreased cell survival. The exact mechanisms behind that remain to be elucidated.

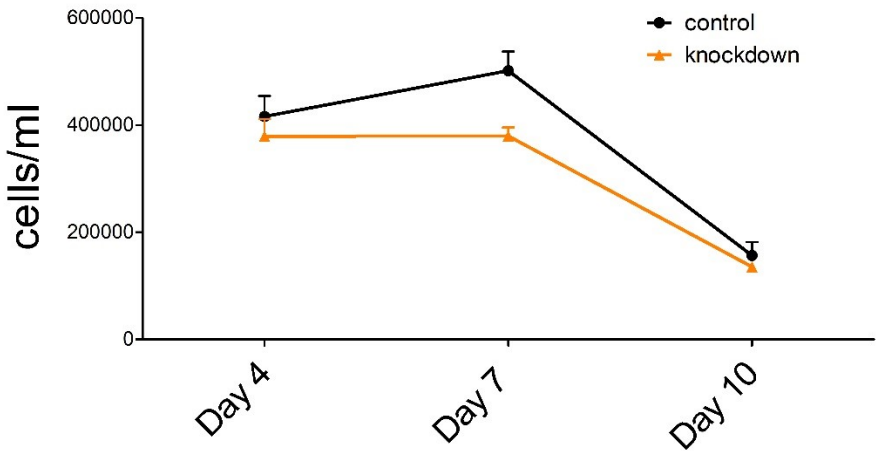


Figure 23: IL-38 knockdown does not increase B cell survival during differentiation
B cells from peripheral human blood were isolated and cultured in a three-step culture system for a total of ten days as indicated above. Knockdown cells were initially incubated with Accell SMARTpool Human IL1F10 siRNA at 1 µM, the control group was treated with Non-Targeting siRNA Pool #1 at 1 µM. At each culture step, the cells were harvested and analyzed by flow cytometry. Living cells were determined by their FSC and SSC characteristics (size and granularity) via flow cytometry. Data are mean concentrations of cells in culture with SEM. n = 3.

5.4.3 IL-38 knockdown seems to reduce B cell immunoglobulin production

Next, we analyzed supernatants from each culture step by ELISA to determine immunoglobulin concentrations. We tested for IgM, IgG and IgA and used optimal dilutions of supernatants for each Ig-subclass (1:10, 1:25, 1:75). In our regular B cell differentiation assay, we observed increased Ig totals of all three subclasses on day 4 of cell culture in cells not being treated with rhIL-38. Looking at the Ig-production per cell and day, these differences could only be confirmed for IgA. Interestingly, IL-38 treated cells showed increased per cell production of IgM on day 10 of the differentiation process. Now, we examined if the reduction of endogenous IL-38 also had a significant influence on Ig-production.

We did not observe statistically significant differences in total IgM concentrations or production per cell and day (**Figure 24A, B**). On day 4, the total IgM concentration was 3218 ng/ml in the control and 2430 ng/ml in the knockdown group. These concentrations were clearly lower than D4 concentrations measured in the regular differentiation assay. This could be a result of the cells initially being stimulated in FCS-free medium, which might have altered IgM secretion. On day 7, the IgM concentrations increased to 34675 ng/ml in the

control and 23280 ng/ml in the knockdown group. Despite the large difference in means, this was not statistically relevant. Besides, the analysis of IgM production per cell and day did not show any changes with 22.47 pg/cell/day compared to 20.47 pg/cell/day. Therefore, the difference in total IgM concentrations on day 7 can be explained by differences in living cells in culture at this timepoint. On day 10, absolute IgM concentrations decreased in both groups compared to day 7, reaching 12128.2 ng/ml in control and 10951.2 ng/ml in knockdown group (not significant).

The total IgG concentrations in supernatants from IL-38 knockdown cells were significantly lower compared to the control group on day 7 of the B cell differentiation ($p = 0.0257$). The control group showed mean IgG concentrations of 279.72 ng/ml on day 4 increasing to 3498.99 ng/ml on day 7 and further to 5456.81 ng/ml on day 10. In the knockdown group, the total IgG concentrations were 233.81 ng/ml on day 4, 2421.14 ng/ml on day 7 and 3460.69 ng/ml on day 10 (**Figure 24C**). Even though the mean difference on day 10 was large, it did not reach statistical significance ($p = 0.0905$). However, this might result from increased cell numbers in the control group as the calculation of IgG production per cell and day did not reveal any significant differences (**Figure 24D**). On day 7, the production was 2.39 pg/cell/day in the control group and 2.09 pg/cell/day in the IL-38 knockdown cells ($p = 0.0715$). On day 10, the mean difference was 3.93 pg/cell/day but showed large variation with a standard deviation of 5.48. Therefore, we only observed a tendency of decreased IgG production in IL-38 knockdown cells appearing on days 7 and 10 of the differentiation process.

For IgA, the results were similar. Day 4 IgA production showed no differences between knockdown and control group in neither total concentrations nor production per cell and day (**Figure 24E, F**). The absolute concentrations in supernatants from day 7 were clearly reduced in the knockdown cells, with means of 2846.77 ng/ml compared to 5005.76 ng/ml in the control ($p = 0.0494$). In production per cell and day at this timepoint, the difference closely missed statistical significance, with 3.33 pg/cell/day in the control and 2.51 pg/cell/day in knockdown cells ($p = 0.0802$). On day 10, the mean differences in total IgA concentrations and in per cell production were 884.05 ng/ml and 1.11 pg/cell/day, both not being statistically significant.

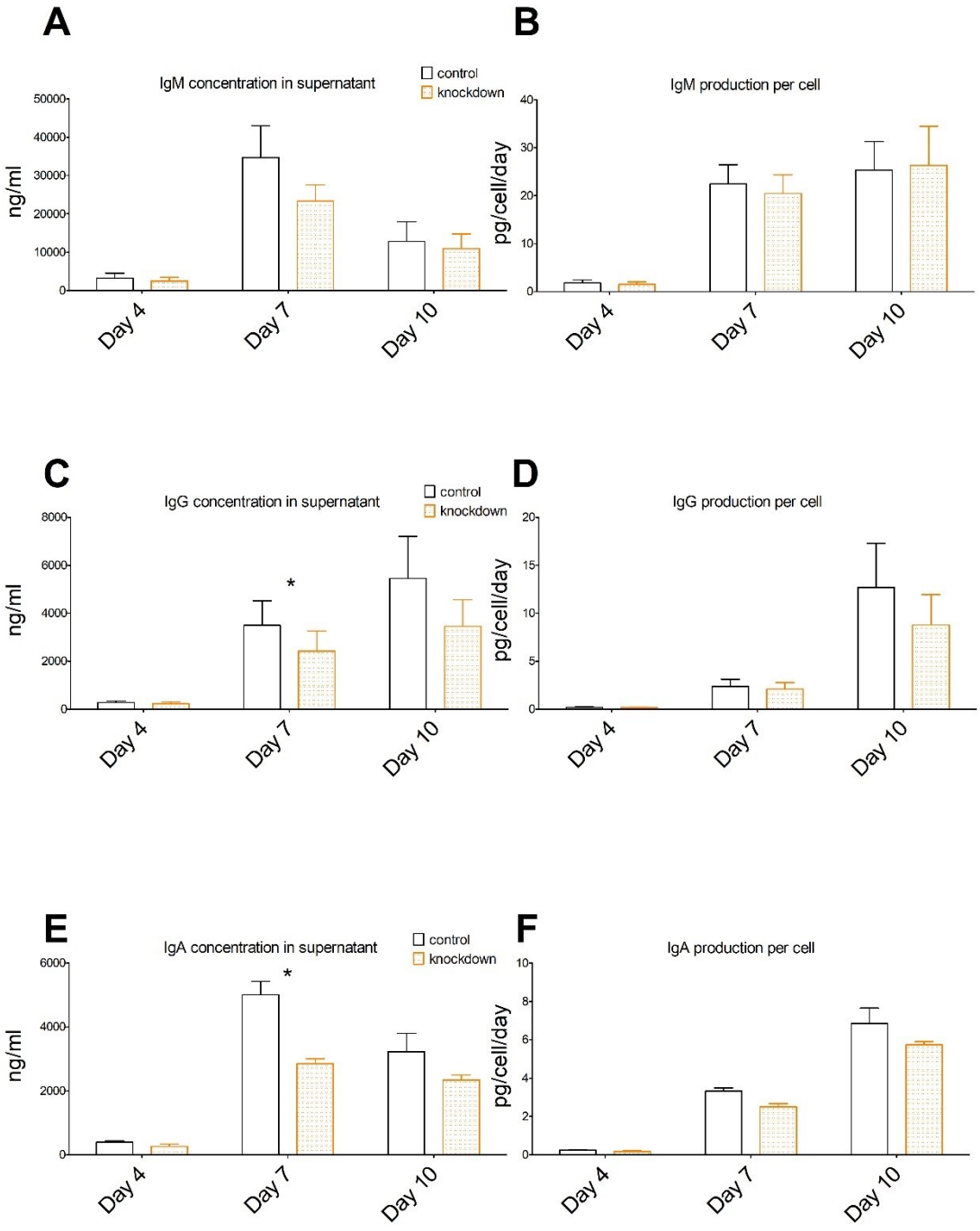


Figure 24: Immunoglobulin production of IL-38 knockdown B cells
B cells from peripheral human blood were isolated and cultured in a three-step culture system for a total of ten days as indicated above. Knockdown cells were initially incubated with Accell SMARTpool Human IL1F10 siRNA at 1 µM, the control group was treated with Non-Targeting siRNA Pool #1 at 1 µM. At each culture step, supernatant was taken and analyzed using Human total Ready-SET-Go!® ELISA kits for IgA, IgG and IgM. Data are means with SEM of immunoglobulin concentrations in cell culture supernatants (**A, C, E**) and immunoglobulin production per cell and day (**B, D, F**). n = 3. *$p < 0.05$.

5.4.4 B cell specific gene expression is not altered by IL-38 knockdown

Finally, we investigated how gene expression levels of B cell specific genes responded to knocking down IL-38 while differentiating naïve and memory B cells into plasma cells. The general progression for all inspected genes was congruent with our previous findings from B cell differentiation protocols with and without IL-21 as stimulant. Comparing control and knockdown cells, no statistical significant differences were found (**Figure 25**). However, all genes showed the same tendency. It appears as on day 4 of the differentiation process gene expression levels in the knockdown group were decreased compared to the control, which was not further evident on days 7 and 10. Unfortunately, due to the low number of replicates profound statistical analysis can not be provided.

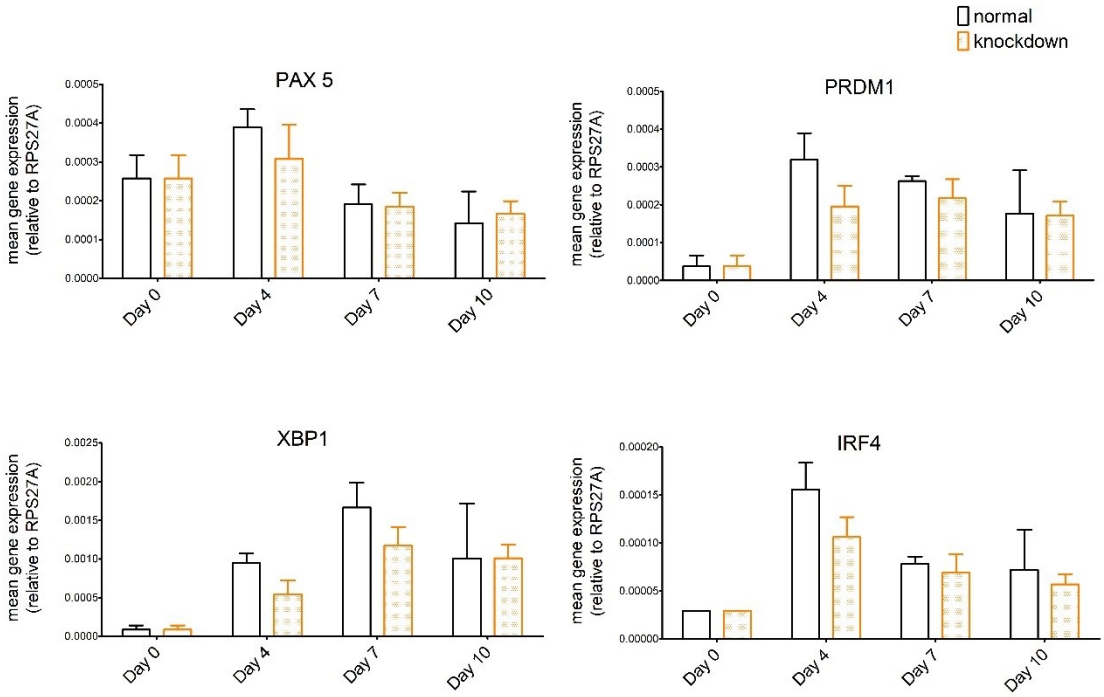


Figure 25: B cell specific gene expression is not altered in IL-38 knockdown cells
B cells from peripheral human blood were isolated and cultured in a three-step culture system for a total of ten days as indicated above. Knockdown cells were initially incubated with Accell SMARTpool Human IL1F10 siRNA at 1 µM, the control group was treated with Non-Targeting siRNA Pool #1 at 1 µM. At each culture step, cells were harvested and washed. RNA expression levels were determined by Real time qPCR. Data are mean gene expression levels relative to RPS27A with SEM. n = 3.

6. Discussion

The group of IL-1 family cytokines plays a central role in the complex construct of regulating innate and adaptive immune responses. The function of one of its members, the relatively young cytokine IL-38, remains to be fully revealed. Since its discovery in 2001 increasing numbers of in vitro and association studies point out an anti-inflammatory effect of IL-38 in immune regulation. However, a potential connection between IL-38 signaling and B cell function, as indispensable part of immunity, has not been investigated. In this project, our aim was to elucidate the influence of exogenous IL-38 on the process of peripheral human blood B cells differentiating into antibody secreting cells. For the first time, we describe direct effects of exogenous IL-38 on differentiating B cells in vitro

1. exogenous IL-38 reduces the transition of CD27+ B cells to CD38+ cells, which is an important phenotypic change in the developmental process towards plasma cells.

2. IL-38 enhances cell death of CD27+ B cells and therewith reduces early antibody production

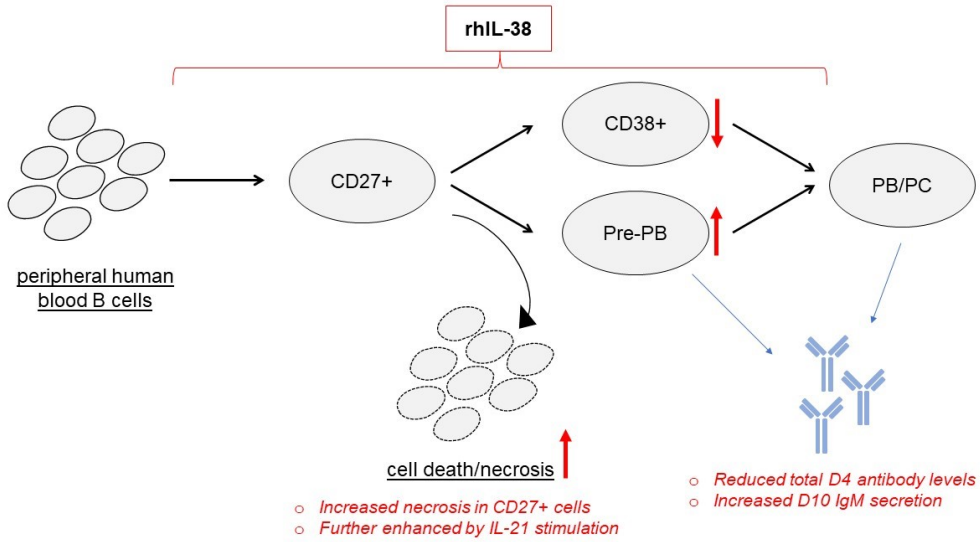


Figure 26: Overview of the effects exogenous IL-38 has on differentiating B cells
Peripheral human blood B cells were stimulated in vitro to differentiate into antibody producing plasmablasts (PB) and plasma cells (PC). During differentiation B cells express CD27+ and further develop through CD38+ cells or antibody producing Pre-Plasmablast (Pre-PB) into PB and PC. The effects of exogenous recombinant human IL-38 (rhIL-38) in this process are displayed in red.

6.1 IL-38 reduces CD38 expression

To this date, only few connections between B cell function and IL-38 have been described. Most importantly, proliferating B cells in tonsils and other lymphatic tissues were shown to express IL-38[5] drawing our interest into investigating the function of IL-38 in B cell activation and differentiation. We examined the influence of exogenous IL-38 in an in vitro model stimulating peripheral human blood B cells to differentiate into antibody secreting cells. Our major finding was IL-38 treatment significantly reduces the CD38 expression on CD27+ B cells. The largest influence was seen on day 7 of the ten-day differentiation process reducing CD38 expression from 38.44% to 33.68%.

The CD38-protein is a transmembrane protein expressed in various tissues and is mostly known as an activation marker of different immune cells, particularly B cells (reviewed in[217]). However, CD38 itself exerts various functions, one as an ectoenzyme crucial in the metabolism of nicotinamide dinucleotide (NAD)[218-220] and second as a receptor expressed on multiple immune cells[221]. Despite CD38 lacking an intracellular signaling domain it has been shown to be involved in cell-cell interactions as coreceptor, for instance with the T cell receptor/CD3, CD31 on endothelial cells and the CD19/CD81 complex on B cells.[222-225] Therefore, CD38 is viewed as an active participator in controlling B cell biology and facilitating activation of mature B cells. In most scenarios, CD38 ligation provides a pro-inflammatory stimulus like promoting chemotaxis and cell activation of the innate and adaptive immune system (reviewed in[226]). Particularly the interaction between B cells and T helper cells seems to be modulated by CD38. T helper cells have been shown to secret cytokines after CD38 ligation, amongst others, IL-6 and IL-10 which are well known to induce plasma cell differentiation in B cells.[227] On B cells themselves, CD38 ligation promotes immunoglobulin secretion and proliferation of mature B cells[228] and enhances proliferation of LPS-activated B cells.[229] Therefore, a reduction of CD38 expression on differentiating B cells by exogenous IL-38 matches the overall perception of IL-38 being an anti-inflammatory cytokine. The transcriptional regulation of CD38 expression has extensively been studied in human airway smooth muscle cells. Here, different elements of transcriptional regulation and binding sites have been described. Two of which are the well-known transcription factors NFκB and AP-1.[230] IL-38

has been shown to bind the IL-1R1 and act antagonistically on its downstream signaling[5,50] which ultimately also affects NFκB and AP-1 expression (reviewed in [21]). Concerning B cell homoeostasis and humoral responses, little is known about direct IL-1 effects and thus IL-1 antagonism in B cell function. The relevance of the IL-1 axis for B cell responses is to date mostly described as indirect effects involving cell types in close proximity to B cells. For instance, follicular T helper and regulatory cells have been shown to be controlled by the poise of IL-1 signaling/antagonism in providing cytokine mediated signals crucial for B cell differentiation (reviewed in[231]). Even though not perfectly reliable in all individual samples, we verified gene expression of the three potential receptors for IL-38 including the IL-1R1 in peripheral and differentiating B cells. This could be a potential connection between IL-38 signaling and CD38 expression as we hypothesize that exogenous IL-38 binds to the IL-1R1 on B cells and antagonizes IL-1R1 dependent NFκB expression, consequently also decreasing CD38 expression. Interestingly, during B cell differentiation, we observed a striking upregulation of the IL-1R1, particularly occurring on day 4 of the differentiation process. IL-38 binding the IL-1R1 and affecting downstream signaling would consequently have more impact a few days into B cell differentiation and would also explain the delayed effects of IL-38 on CD38 expression. Conveniently, in the experiments with additional IL-21 stimulation the upregulation of IL-1R1 was considerably reduced, leaving a possible explanation for less significant CD38 expression differences between control and IL-38 treated cells in this experimental setting.

Another possible explanation why the phenotypic changes were most prominent on day 7 analysis is the timepoint of IL-38 application itself. IL-38 was applied when seeding the cells and then daily between day 3 and 7. Given the half-life of IL-38 at about 7 hours,[8] possibly a daily application of IL-38 is necessary to ensure consistently effective IL-38 stimulation in culture. Consequently, the largest phenotypical changes occurred after four consecutive days of IL-38 treatment. This theory is supported by the again decreasing differences in B cell phenotype towards day 10. More importantly, exogenous IL-38 mostly seems to affect B cells which have acquired CD27+ phenotype, as we exclusively found CD38 expression on cells already expressing CD27. In our approach, we used

all B cell subsets found in peripheral human blood. Therefore, in the beginning of the differentiation process, a mean 21.57% of all B cells expressed CD27, predominantly being memory B cells. After initiating differentiation, we first observed an increase in CD27+ cells, before creating relevant numbers of CD38+ cells. This could explain, why exogenous IL-38 does not influence the first days of B cell differentiation, as CD38 expression mostly occurs later in the differentiation process.

Apart from IL-1 signaling, increasing evidence suggests IL-38 to act antagonistically on the IL-36R pathway similar to the IL-36Ra. However, as described for the IL-1 axis, the exact influence of IL-36 signaling in B cell homoeostasis is also not well examined. In this study we confirmed the presence of IL-36R on B cells on level of gene expression. In comparison to IL-1R1 and IL-1RAPL1, mRNA expression of the IL-36R was reliably detectable in all conducted experiments and through all stages of B cell differentiation. In experiments with IL-36R deficient mice, Schmitt has shown that peripheral B cell subsets in the spleen, as well as formation of germinal centers are not affected by IL-36R deficiency indicating a low importance of the IL-36 axis in B cell differentiation. However, they also showed B cells to react to IL-36α stimulation with increased IL-6 and TNF-α secretion depicting an autocrine survival signal for B cells.[232] Furthermore, unstimulated B cells themselves produce IL-36 cytokines including IL-36Ra and in plasma cells IL-36α production is clearly increased.[232] Consequently, IL-36 signaling seems to be important in regulating B cell and plasma cell function at least by influencing surrounding cells. Schmitt et al. depicted IL-36 signaling to mediate the communication between B cells and fibroblast like synoviocytes (FLS) as plasma cells cocultured with IL-36R deficient FLS showed clearly reduced survival.[233] Therefore, they concluded that plasma cell survival in specialized niches is mediated by IL-36R dependent signaling. Hence, IL-38 as antagonist of the IL-36R pathway must be considered as adjusting element in plasma cell homoeostasis. Further studies are required to prove the binding of IL-38 on the aforementioned B cell receptors on a protein level and should include analysis of downstream transcription factors like NFκB to support these hypotheses. Additionally, other immune cell types known to

provide crucial signals for differentiating B cells should be included in these examinations and closely investigated in their susceptibility to exogenous IL-38.

In this study, our focus of gene expression analysis was put on B cell specific genes known to be crucial for plasma cell development. We did not observe significant differences for PAX5, PRDM1, XBP1 or IRF4 during the first 7 days of the differentiation process. However, on day 10, IRF4 expression was significantly elevated by IL-38 treatment. Increased IRF4 expression is generally associated with enhancing plasma cell development and in this course also increasing CD38 expression and therefore cannot explain the phenotypical differences we observed.

In the final days of the differentiation protocol CD38 expression further increased, while the share of memory B cells (MBCs) remained constant and the group of pre-plasmablasts (Pre-PB) decreased. It therefore appears as Pre-PB constitute the major contributor to CD38+ cells and ultimately plasma cells during the final phase of differentiation. Interestingly, IL-38 treatment increased the share of Pre-PB. Jourdan et al. described the population of Pre-PB as population occurring during differentiation of MBCs towards antibody secreting plasma cells.[213] This could indicate that IL-38 does not suppress plasma cell generation in general, but rather stimulates activated B cells to differentiate into Pre-PB instead of directly towards plasmablasts and plasma cells. As there exist different pathways of plasma cell development, this could also explain, why ultimately, we did not find significant differences in plasma cells and the share of cells defined as MBCs is also not affected by exogenous IL-38. To this point, the biological relevance of Pre-PB is not understood. As Pre-PB secrete immunoglobulins at lower levels than plasmablasts or plasma cells, it seems plausible that driving B cells into this transitional state also perpetuates an immune regulatory effect.

6.2 IL-38 affects immunoglobulin production and promotes cell death

After finding phenotypical differences caused by exogenous IL-38 treatment, we next analyzed immunoglobulin production of the B cells. In cells treated with exogenous IL-38, we observed a significant reduction of total Ig concentrations of all Ig-subclasses after 4 days of the differentiation process. This decrease

however could not be verified on a single cell production level. Hence, we believe that this reduction is predominantly caused by tendentially decreased numbers of living cells in the treatment group, instead of cellular mechanisms impacting the production of immunoglobulins. Nevertheless, we also observed increased IgM production per cell for day 10 B cells treated with IL-38. Manjarrez-Orduño et al. reported that CD38 stimulation of LPS activated B cells decreases the development of IgM producing plasma cells.[229] Therefore, it seems plausible that decreased CD38 expression on IL-38 treated B cells comes along with increased IgM production. The underlying mechanism of this observation has not been finally revealed. Manjarrez-Orduño described a Blimp-1/PRDM1 dependent reduction of IgM producing plasma cells, which we did not observe in our experiments. Another explanation would be exogenous IL-38 decreasing isotype-switching and consequently leaving an increased percentage of IgM producing cells. In this case however, we would also expect significant differences in the production levels of the other Ig-subclasses, which were not evident. Altogether it seems plausible that exogenous IL-38 via reduction of CD38 expression and further unknown downstream signals enhances IgM production of activated B cells. The biological relevance behind that must remain unclear.

The observation of reduced living cell counts in the group of IL-38 treated B cells, which affected immunoglobulin levels, raised our interest in investigating the aspect of cell death more closely. In our proliferation and cell death assay, we then confirmed, that exogenous IL-38 decreases the survival of B cells and increases the percentage of necrotic cells. These findings were predominantly evident in CD27 expressing cells. We were surprised by the fact that IL-38 treatment caused necrosis of B cells, as necrosis is known as unregulated cell death enhancing inflammatory responses and IL-38 was expected to act anti-inflammatory. However, these necrotic cells are most likely secondary necrotic cells originating from primary apoptotic cells. Secondary necrosis occurs when apoptotic cells are not cleared before their cell membrane destabilizes.[234,235] In our in vitro setting, no professional phagocytic cells were present which could have eliminated apoptotic B cells. Therefore, we conclude that IL-38 may increase B cell mortality by promoting apoptosis of activated CD27+ B cells.

So far, connections between IL-38 and induction of cell death mechanisms remain rare. Pro-apoptotic effects of IL-38 have been described first by Wang et al. in 2018, showing IL-38 to have a protective function in non-small cell lung cancer (NSCLC) by increasing apoptosis of NSCLC cells.[236] Recently, another group has shown IL-38 to promote apoptosis of colorectal cancer cells by inhibiting the ERK-signaling pathway.[237] Apart from that, apoptotic cancer cell lines have been shown to release high amounts of IL-38 exerting an anti-inflammatory effect on human macrophages.[9] We did not examine if apoptotic B cells also secrete IL-38. To improve our knowledge concerning IL-38 and B cell survival further investigations are needed. For one, it would be interesting to also investigate cell death in the proportion of CD38 expressing cells, as it seems possible that exogenous IL-38 particularly decimates CD38+ cells by inducing apoptosis. This is supported by the fact, that in the regular differentiation assay, differences in CD38 expression were not evident before day 7 and fittingly, significant disparities in cell survival were also evident on this point of analysis. The pathway through which IL-38 could exert an apoptotic effect remains unclear and requires more detailed research. Interestingly, Zupo et al. have shown that CD38 ligation is able to prevent apoptosis in germinal center B cells.[238] Additionally, in B cell malignancies such as Chronic Lymphatic Leukaemia (CLL), CD38 expression seems to contribute to the survival advantage of degenerated cells and is viewed as negative prognostic marker for CLL patients.[239] Consequently, it also seems possible that IL-38 does not directly promote apoptosis of B cells but the reduction of CD38 expression secondly increases B cell mortality. Further functional analyses are needed to confirm this theory.

6.3 Methodological limitations and the influence of IL-21

The nature of plasma cell development and homoeostasis in vivo is highly complex and diversely regulated. It requires multiple organ systems and unique cellular niches with numerous cell types and stimulation factors involved. To perform basic research on such a complex structure is challenging and always limited to a certain extent of biological truth. In this study we established a three-step cell culture system to mimic B cell to plasma cell development in humans. Our approach naturally brings certain limitations concerning the transferability of its results to in vivo biology. For one, in our broad approach we used B cells from

peripheral human blood. Consequently, we started with a heterogenous group of cells, which given the observation that IL-38 mostly affects CD27+ cells, might decrease the measured outcome. It would be interesting to perform a similar experiment using pre-sorted CD27+ B cells. The fact that IL-38 expression was particularly found in specialized lymphatic tissues like spleen and lymph nodes supports the idea that its highest B cell specific relevance is in a context of pre-activated CD27 expressing cells found in these tissues at higher percentages compared to the compartment of peripheral human blood.[240] A high percentage of naive B cells as found in human blood would therefore reduce possible effects of exogenous IL-38. Furthermore, in our experiments the expression levels of CD38 took center stage of IL-38 induced changes. As mentioned before, the biological relevance of CD38 goes far beyond than being an activation marker for B cells but influences cell-cell interactions for instance with T-helper cells. In our in vitro differentiation protocol, T cell interaction was mimicked by use of cytokines only and cannot fully simulate in vivo cell interactions. Therefore, a small reduction of CD38 expression in vitro might have even greater effects in vivo by also affecting B cell – T cell interactions. Studies using co-culture systems with T cells and B cells would be interesting to further address this question.

Another issue in our experiments was the high amount of cell death, which is a well-known obstacle of in vitro B cell research. Despite the diverse stimulation protocol, cell death occurred at high rates and particularly in the final phase of the protocol the percentage of vital cells decreased clearly. It is to assume that this also influences the overall experimental outcome and observed changes, especially as the percentages of created plasma cells were lower than initially expected. The quantity of acquired cell material towards the end of the differentiation assay is also relevant for qPCR analysis as it affects quality of RNA isolation. With reduced cell viability the quality of the qPCR data was not constantly satisfying and therefore may be questioned. For future research, higher overall cell counts would be desirable to improve the quality of qPCR analysis.

We tried to address the problem of high cell death rates by adding IL-21 to the differentiation protocol, as it is known to be a potent enhancer of B cell differentiation, proliferation, and plasma cell development. Indeed, this clearly

produced increased percentages of differentiated B cells and higher immunoglobulin production, but the overall concentration of cells in culture was not altered by IL-21. Ultimately, the addition of IL-21 seemed to reduce phenotypic changes created by exogenous IL-38 but potentiated the pro-apoptotic effect of IL-38. This could indicate that IL-38 only plays a minor role as regulator of B cell differentiation, and in this in vitro protocol is undermined by the strong effect IL-21 exerts on B cell differentiation. In fact, IL-21 is widely recognized as cytokine exerting the strongest pro-differentiation stimulus on various B cell subsets.[195,241,242] This is also supported by the fact that the group of Pre-PB was not distinguishable in experiments using IL-21 suggesting that IL-21 treatment outweighs stimulatory effects of other cytokines consequently altering B cell compositions. Concerning the aspect of cell death, IL-21 is known to be able driving B cells into apoptosis depending on costimulatory signals.[243-245] We observed that IL-21 treatment alone clearly increased rates of apoptosis in CD27- B cells. However, the combination of IL-21 and IL-38 preponed and increased cell death of CD27+ cells. As mentioned before, you could hypothesize that increased cell death by exogenous IL-38 might be caused by a reduction of CD38 expression and further downstream effects. Yet, these observations suggest otherwise as differences in CD38 expression were reduced by adding IL-21 and particularly on day 4, where cell viability is already significantly affected, phenotypical changes were not evident to support this theory. Therefore, it seems more plausible that further mechanisms are involved where IL-38 and IL-21 synergistically promote apoptosis of certain B cells. Either way more detailed research concerning apoptosis-mediating signals are required concerning both cytokines.

6.4 IL-38 knockdown experiments

At last, we established an IL-38 knockdown experiment to examine a possible internal function of IL-38 in differentiating B cells. We were able to create a satisfying knockdown significantly reducing IL-38 mRNA expression of day 4 B cells to 44% compared with respective control cells. We then again analyzed cell phenotypes, immunoglobulin production levels and cell viability between IL-38 knockdown and control cells during B cell differentiation.

Interestingly we observed a clear tendency of elevated CD38 expression in IL-38 knockdown cells on day 4 of the differentiation process. This was striking to us, as in all experiments using exogenous IL-38, we had observed the opposite with a reduction in CD38 expression. Therefore, these results not only suggest that IL-38 also has internal functions in B cell differentiation regulation but further support our findings from the previous experiments suggesting IL-38 to act immune-regulatory by reducing CD38 expression in activated B cells. As B cells have been shown to be a major source of IL-38 production among human PBMCs[10] it is to discuss if the IL-38 knockdown and therewith reduction of IL-38 secretion simply results in decreased exogenous IL-38 signaling. Given the results of our previous findings, the construct of an autocrine IL-38 stimulation loop would explain the tendency of increased CD38 expression in IL-38 knockdown cells. However, speaking against this theory is the fact that the IL-38 knockdown also affected the composition of CD27+ cells as well as naïve B cells seen on day 7 analysis. As our previous experiments congruently showed no effect of exogenous IL-38 on these phenotypes it seems unlikely being a consequence of reduced autocrine IL-38 stimulation. To further address this question, we tried to measure IL-38 protein concentrations in supernatant from the B cells but could not reliably detect IL-38 (data not shown). Possibly, cell counts in our experiments were too low to create detectable concentrations of secreted IL-38. Future research should include higher cell counts or improved IL-38 detection methods to further investigate possible autocrine stimulation loops in B cell differentiation. Besides, the usage of an IL-38 blocking antibody could be useful in this scenario.

Analyzing immunoglobulin production, a tendency of reduced IgG and IgA production in IL-38 knockdown cells was visible. This was also unexpected, as in a general anti-inflammatory perspective of exogenous IL-38 you could suspect dysregulated and increased Ig production in IL-38 knockdown cells. However, as concerning the cell phenotypic changes it seems that the endogenous functions of IL-38 are difficult to predict and differ from conclusions drawn of its external functions. For instance, Huard et al. have recently reported that IL-38 deficient mice show reduced aspects of experimental autoimmune encephalomyelitis with decreased inflammatory cytokine production and altered cell metabolism in bone

marrow derived macrophages.[246] These results were also unexpected given the knowledge of the predominantly anti-inflammatory effects exogenous IL-38 exerts. Therefore, we must recognize that the endogenous function of IL-38 is still poorly understood but seems to affect internal processes like cell differentiation and immunoglobulin production of B cells.

Unfortunately, as one sample had to be declared an unsuccessful knockdown, the replicates in our knockdown experiments were generally low and therefore lack statistical profoundness and significance. It is to speculate if a higher number of replicates would have brought more significant results. Also, the largest effect by the IL-38 knockdown was evident on day 4, accompanied by the greatest reduction in IL-38 mRNA expression. As we only applied the siRNA once in the beginning of the differentiation process, successful suppression of IL-38 expression did not go beyond day 4 analysis. Therefore, a prolonged and even more sufficient IL-38-knockdown could have even larger impact on cell phenotypes during B cell differentiation.

For B cell specific gene expression and cell counts during the knockdown experiments, no significant differences between the two groups became evident. That further indicates that the endogenous function of IL-38 compared to the exogenous function overall has less relevance for differentiating B cells. Concerning the aspect of cell death, one may conclude that the pro-apoptotic effect of IL-38 is strictly limited to an exogenous effect. Considering what is currently known about IL-38, that it is secreted by other apoptotic cells[9,15] and the recent description of a pro-apoptotic signaling through the ERK-receptor pathway[237] support this theory.

6.5 Concluding remarks and outlook

Over the last years, increasing effort has been put into investigating the biological function of the relatively young cytokine IL-38. Increasing evidence shows direct relations between IL-38 and various autoimmune diseases. Some groups have even used IL-38 as therapeutic agent in murine models of Psoriasis,[17] RA[50] and SLE[95] each with promising results. For the first time, we now examined an immediate impact of IL-38 on B cells, which depict an indispensable part of immunity and therefore also autoimmune disorders. We found exogenous IL-38

to reduce the differentiation of peripheral human blood B cells into antibody producing cells and to increase apoptosis of pre-activated CD27+ cells in this process. Additionally, the early secretion of antibodies is also reduced by exogenous IL-38. Unfortunately, the exact underlying mechanisms and signal transductions remain unknown at this point. Nevertheless, our study joins other reports pointing out a B cell relevant function of IL-38, which could be helpful understanding pathogeneses of various autoimmune disorders which are associated with IL-38. For instance, IL-38 serum levels are elevated in patients with SLE and correlate with disease activity and severity.[92] Also, B cell homoeostasis is altered in patients with SLE. In 2000 Odendahl et al. already showed the presence of a general B cell lymphopenia in SLE patients with emphasis on mature naïve B cells. At the same time more differentiated B cells, which had already been in contact to antigens, like memory B cells, plasmablasts and plasma cells were found to be significantly increased in peripheral human blood.[247] Now, given our findings, you could hypothesize that IL-38 levels and altered B cell compositions in SLE might somehow be connected. Interestingly, de Graaf et al. had shown that peripheral human blood B cells are the major source of IL-38 production in human blood.[10] Further, different studies have reported that silencing IL-38 in human PBMCs results in increased production of cytokines like IL-6 and APRIL, which are not only very well correlated with SLE pathogenesis but also known as potent enhancers of B cell differentiation.[92] Therefore, one could conclude that elevated IL-38 in autoimmune disease like SLE is a self-regulatory mechanism to reduce inflammation and the formation of antibody secreting cells. Our findings concerning the ability of IL-38 to induce apoptosis of B cells can also be factored in as possible explanation of B cell lymphopenia in SLE. In various further autoimmune disorders, IL-38 has also clearly been correlated with disease. For instance, IL-38 serum levels are increased in patients with RA[11] which could also be a counter regulatory mechanism in a dysregulated steady state of immune cell activation. This is also supported by the interesting findings of IL-38 in sepsis. Xu et al reported that inhibiting IL-38 increased mortality in experimental sepsis, while the administration of IL-38 even improved survival and reduced production of pro-inflammatory cytokines.[110] As mortality in sepsis predominantly descends from dysregulated and overwhelming immune responses with following organ

damages, this study clearly outlines the anti-inflammatory properties of IL-38 and its role in immune regulation. In other diseases like psoriasis, IL-38 might even be part of its emergence, as IL-38 expression in psoriatic skin is significantly decreased,[17] which can be interpreted as component of its pathogenesis in pro-inflammatory stimuli. However, in all aforementioned diseases, specific connections remain exceptional.

The study at hand, now gives first insights into direct effects of IL-38 on B cells, but essential questions remain to be answered. First, further investigations are required showing the exact receptors and respective signaling pathways of IL-38 on B cells. For this purpose, genetic analysis of downstream signals of the three known IL-38 binding receptors, IL-1R1, IL-36R and IL-1RAPL1, after IL-38 stimulation could generate new knowledge. Same applies for molecular mechanisms of the pro-apoptotic effect IL-38 has on B cells. We would suggest to first focus on more differentiated B cells such as memory B cells or even follicular B cells obtained from lymph nodes, as in our experiments CD27+ cells were most affected by exogenous IL-38. Subsequently, other B cell subsets and immune cells in direct context of B cell homoeostasis should be tested on their susceptibility to IL-38. Eventually, at this point it seems like B cells are a main producer of IL-38 in human blood and in specialized lymphatic tissues. The underlying purpose and signals behind that remain unknown. Possibly, B cells produce IL-38 as a self-regulating mechanism to decrease the differentiation of antibody secreting cells. However, it seems biologically consistent that other immune cells in close proximity to B cells are also affected by IL-38. Therefore, the compartments of secondary lymphatic tissues and their respective cellular components also depict a promising field for more detailed research of IL-38 and B cell homoeostasis.

Ultimately, IL-38 is already connected to numerous diseases that are highly relevant for modern society, particularly cancerous and autoimmune pathologies. Our study now draws a connection between IL-38 directly impacting human B cells and paves way for more detailed investigations that hopefully one day lead to the diagnostic or therapeutic usage of IL-38 in disease.

7. References

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8. Publications

Huard, Arnaud*; Wilmes, Christian*; Kiprina, Anastasiia; Netzer, Christoph; Palmer, Gaby; Brüne, Bernhard; Weigert, Andreas (2023): Cell Intrinsic IL-38 Affects B Cell Differentiation and Antibody Production. In: *IJMS* 24 (6), S. 5676. DOI: 10.3390/ijms24065676

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9. Acknowledgement

10. Lebenslauf

11. Schriftliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel

The influence of IL-38 in the process of B cell differentiation

In dem Institut für Biochemie I unter Betreuung und Anleitung von Prof. Dr. Andreas Weigert ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Thesis angeführten Hilfsmittel benutzt habe. Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht*. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

Vorliegende Ergebnisse der Arbeit wurden (oder werden) in folgendem Publikationsorgan veröffentlicht:

Huard, Arnaud*; Wilmes, Christian*; Kiprina, Anastasiia; Netzer, Christoph; Palmer, Gaby; Brüne, Bernhard; Weigert, Andreas, Cell Intrinsic IL-38 Affects B Cell Differentiation and Antibody Production, International Journal of Molecular Sciences, 24 (6), S. 5676, 2023

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(Ort, Datum) (Unterschrift)