Role of CR3 (CD11b/CD18) and CR4 (CD11c/CD18) expressed by B lymphocytes of healthy donors and chronic lymphocytic leukaemia patients

Ph.D. Thesis

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List of abbreviations

ABC	Age Associated B Cell
ADMIDAS	adjacent to MIDAS
ANOVA	ANalysis Of VAriance
APC	AlloPhycoCyanin
ATCC	American Type Culture Collection
BCR	B Cell Receptor
BTK	Bruton's Tyrosine Kinase
βTD	β-Tail Domain
CD	Cluster of Differentiation
CD40L	CD40 Ligand
cDNA	Complementary DNA (DeoxyriboNucleic Acid)
CHO cell line	Chinese Hamster Ovary cell line
CLL	Chronic Lymphocytic Leukaemia
CpG	Short single-stranded synthetic DNA molecules that contain a cytosine triphosphate deoxynucleotide ("C") followed by a guanine triphosphate deoxynucleotide ("G"). The "p" refers to the phosphodiester link between consecutive nucleotides.
CpG-ODN	CpG OligoDeoxyNucleotide
CR3	Complement Receptor 3
CR4	Complement Receptor 4
CXCR	C-X-C Chemokine Receptor
DAPI	4′,6-DiAmidino-2-PhenylIndole
DC	Dendritic Cell
EBV	Epstein-Barr Virus
EDTA	EthyleneDiamineTetraacetic Acid
EGF	Epidermal Growth Factor
FAK	Focal Adhesion Kinase
Fc	Fragment crystallizable
FCS	Fetal Calf Serum
FDC	Follicular Dendritic Cell
FITC	Fluorescein IsoThioCyanate
FOXP3	Forkhead box P3
GC	Germinal Center
GTPase	Guanosine TriPhosphatease
GUSB	Gene of β-glucuronidase
I-domain	Inserted domain
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
IL2RG	Interleukin 2 Receptor Subunit Gamma

IL9R	Interleukin 9 Receptor
ITGAM	Gene of Integrin Subunit Alpha M
ITGAX	Gene of Integrin Subunit Alpha X
LFA-1	Lymphocyte Function-Associated antigen 1
Mac-1	Macrophage-1 antigen (CR3)
MFI	Mean Fluorescence Intensity
MIDAS	Metal-Ion-Dependent Adhesion Site
mRNA	Messenger RNA
NK cells	Natural Killer cells
OGT	O-Linked N-Acetylglucosamine Transferase
p130Cas	p130 Crk-associated substrate
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PE	PhycoErythrin
PLL-PEG	Poly (L-Lysine)-graft-Poly (Ethylene Glycol)
PMA	Phorbol Myristate Acetate
PSI domain	Plexin-Semaphorin-Integrin domain
PTB domains	PhosphoTyrosine-Binding domains
Rap1	Ras-proximate-1
RapL	Rap-ligand
RGD	Arginine-Glycine-Aspartate
Rho GTPase	Ras homolog GTPase
RIAM	Rap1-interacting adaptor molecule
RMFI	Relative Mean Fluorescence Intensity
RNA	RiboNucleic Acid
RQ-PCR	Real-time Quantitative Polymerase Chain Reaction
SD	Standard Deviation
SDF-1	Stromal cell-Derived Factor-1
SLE	Systemic Lupus Erythematosus
TLR	Toll-Like Receptor
TM	TransMembrane
VLA	Very Late Antigen

1. Introduction

Complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18) belong to the family of β_2 integrins which are widely expressed on myeloid cells where they are known for long to participate in actin linked functions like phagocytosis, adhesion, and migration. These receptors can also be detected on certain subpopulations of B cells or after activation by different stimuli (1–6). Moreover, these complement receptors have been shown to be expressed in various B cell malignancies such as in chronic lymphocytic leukaemia (CLL) (7–14). The kinetics of expression and the role of these β_2 integrins however, in human B lymphocytes have only scarcely been studied so far.

In my thesis I will present our results which extend our knowledge regarding the expression and role of CR3 and CR4 on B cells of healthy donors (15) and chronic lymphocytic leukaemia patients (16), and discuss their relevance in normal immune response and in disease pathomechanism.

1.1. Integrins

Integrins are cell adhesion molecules that play critical role in cell-cell, cell-extracellular matrix, and cell-pathogen interactions, controlling the response to different environmental stimuli. The name "integrin" was given to highlight the importance of these molecules for linking the extracellular matrix to the cytoskeleton (17,18). These receptors mediate actin-linked functions like adhesion and migration, thereby they are involved in various steps of immune responses such as leukocyte trafficking, immunological synapse formation, co-stimulation and phagocytosis.

Regarding their structure, integrins are hetero-dimeric glycoproteins composed of non-covalently associated α and β subunits. In vertebrates there are 18 α and 8 β subunits that can assemble into 24 different heterodimers, each with a specific, nonredundant function determined by their different ligand-binding specificities and diverse tissue distribution (19). Thereby they can be categorized into subgroups based on their subunit composition, ligand-binding properties and in the case of β_2 and β_7 integrins, their restricted expression on white blood cells (**Figure 1**) (19,20).
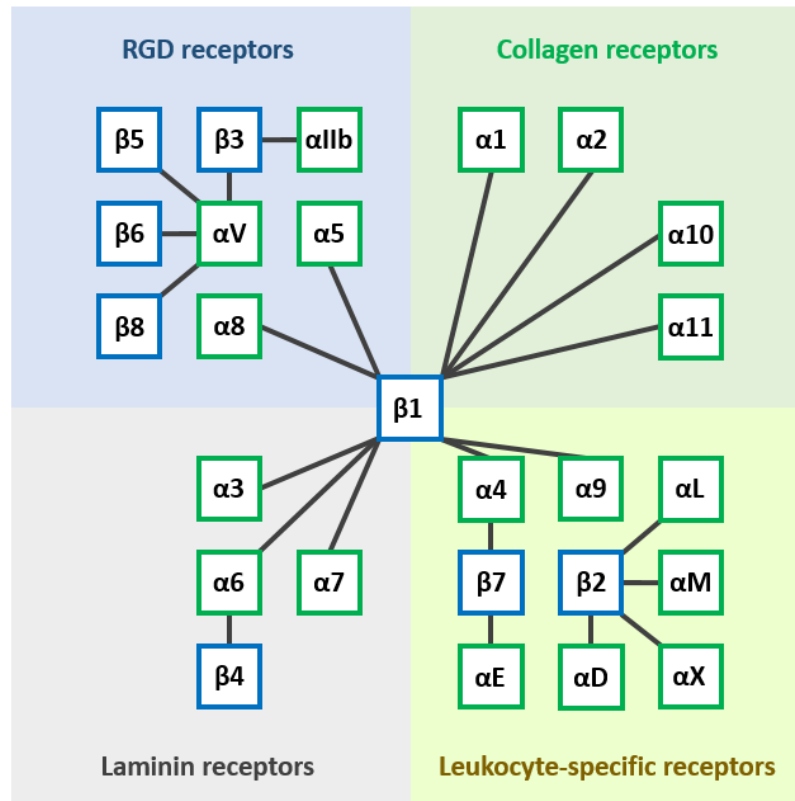
Figure 1. The conventional categories of integrins. The four subgroups are: RGD receptors, Laminin receptors, Collagen receptors, and Leukocyte-specific receptors.

There are four commonly used categories of integrins: i./ the RGD receptors, ii./ laminin receptors, iii./collagen receptors and iv./ leukocyte-specific receptors. RGD receptors recognize the Arg-Gly-Asp (Arginine, Glycine, and Aspartate) tripeptide sequence in molecules such as fibronectin and vitronectin, whereas laminin receptors mediate adhesion to basement membrane laminins. While these two subfamilies are evolutionary ancient, the other two can be found only in vertebrates. Out of these, collagen receptors (α_1, α_2, α_{10}, α_{11}) and the related $\alpha_4\beta_1$ and $\alpha_9\beta_1$ integrins are known to bind extracellular matrix proteins and immunoglobulin (Ig) superfamily cell surface counterreceptors. Finally, the expression of leukocyte-specific integrins is restricted to immune cells where they recognize numerous ligands such as Ig-superfamily counterreceptors or complement fragments, and mediate various immune-related functions (19,20).

1.2. Complement receptors CR3 and CR4

CR3 (CD11b/CD18, $\alpha_M\beta_2$, also known as macrophage-1 antigen (Mac-1)) and CR4 (CD11c/CD18, $\alpha_X\beta_2$, also designated as p150,95) belong to the family of leukocyte-specific receptors. They are named complement receptors after their first identified ligand, which is the complement activation fragment, iC3b (21,22). Beside this protein they are able to bind a wide variety of different structures such as intercellular adhesion molecules, lipopolysaccharides, fibronectin or fibrinogen (23–25). These integrins comprise CD18 (β_2), which is the β subunit and CD11b (α_M) or CD11c (α_X) which is the α chain of the heterodimer (**Figure 2**).

Figure 2. General structure of β_2 integrins (Tan 2012, Erdei 2019 (26,27), modified). CR3 and CR4 belong to the family of β_2 integrins, which are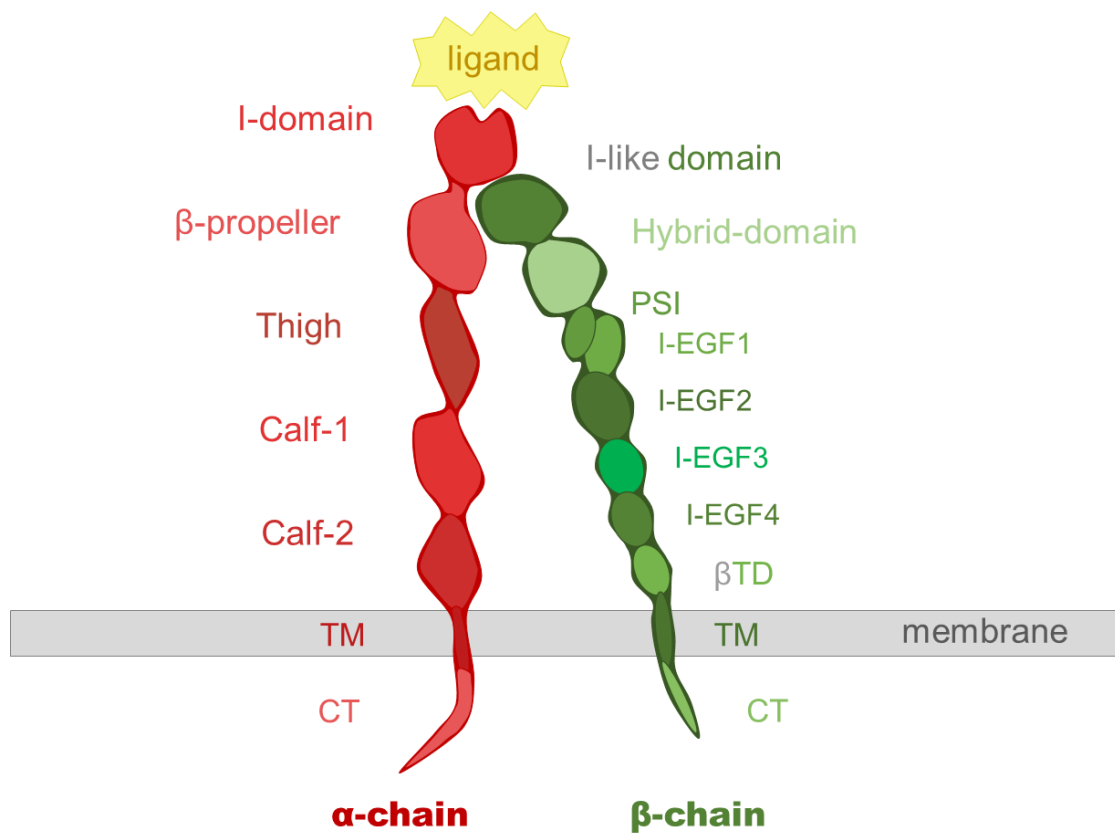 composed of the CD18 (β_2) β chain and the CD11b (α_M) or CD11c (α_X) α chains. Both subunits consist of a long extracellular part, a short transmembrane (TM) domain and a cytoplasmic tail (CT).

The α subunit is composed of the following extracellular segments: "inserted" or I-domain, a seven-bladed β-propeller, a thigh, a calf-1, and a calf-2 domain, followed by a short transmembrane region and the α cytoplasmic tail. The αI-domain, which is composed of approximately 200 amino acids, can be found between blades 2 and 3 in the β-propeller (28). The ligand binding of the integrins is dependent on bivalent cations. At the "top" of the αI-domain there is a Mg^{2+} coordination site called the metal ion-dependent adhesion site (MIDAS) (19). This domain forms the ligand recognition part of the receptor. EF-hand domains are located on the last four blades of the β-propeller and bind Ca^{2+} ions on the lower side, facing outward from the ligand-binding surface of the receptor. Ca^{2+} binding to these sites is suggested to have a role in regulating integrin activation that allosterically affects ligand binding (20,26).

The extracellular part of the β-chain consists of an N-terminal βI or I-like domain, a hybrid domain, a cysteine-rich plexin-semaphorin-integrin (PSI) domain, four cysteine-rich epidermal growth factor (EGF) repeats (I-EGF1, -2, -3, -4) and a β-tail domain (βTD). This structure is followed by a single transmembrane region and a short cytoplasmic tail. Similarly to the αI-domain, the βI-subunit also contains a Mg^{2+} coordinating MIDAS that serves to bind ligands. The site adjacent to MIDAS (ADMIDAS) regulates the MIDAS - by binding an inhibitory Ca^{2+} ion. This ADMIDAS site also binds a Mn^{2+} ion. The cytoplasmic tail of the β subunit contains NPX/Y motifs that can bind proteins containing phosphotyrosine-binding (PTB) domains (20,26).

1.2.1. Inside-out signalling

The integrins have three possible conformations which are related to the ligand binding affinity of the receptor: i/ bent, low affinity/inactive, ii/ extended with intermediate affinity and iii/ extended, open conformation, with high affinity (29–31)(**Figure 3**). Integrins are usually in their inactive, bent conformation on the cell surface, which allows free circulation of leukocytes in blood vessels. In this form both subunits are positioning the I-domain towards the cell membrane. Activation of integrins is regulated by inside-out signalling following intra- or extracellular stimuli, which induces the separation of the α and β cytoplasmic tails. This process is triggered by cytosolic proteins connecting integrins to the actin cytoskeleton (26) inducing a 'switchblade-like' conformational change (29,32). This results in the active, extended state of the integrins, in which they are able to bind their ligands and fulfil their various functions (27).
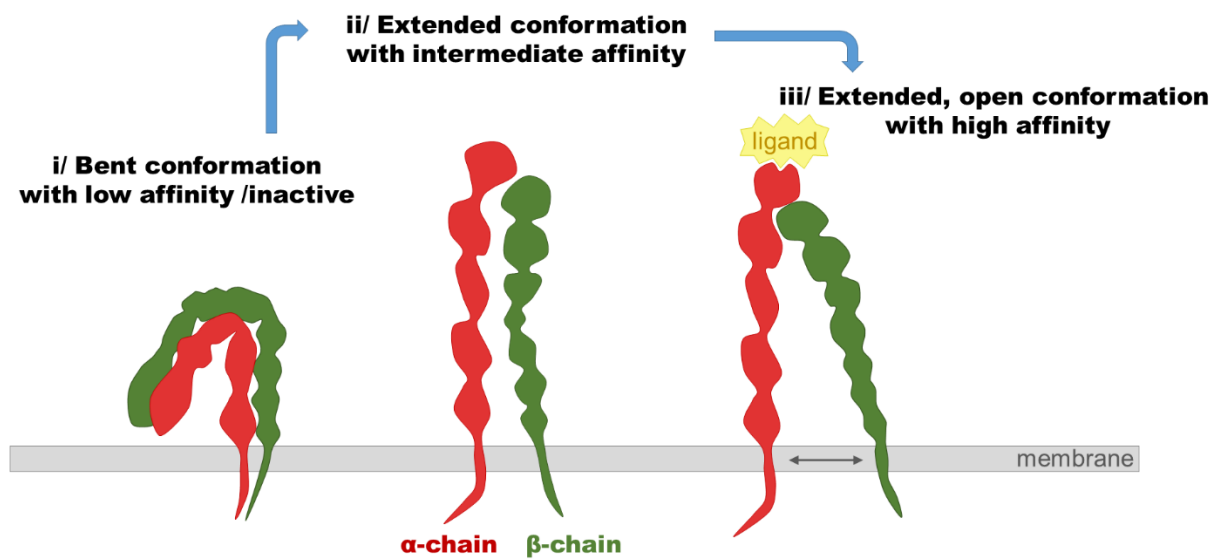
Figure 3. Conformational changes of β₂ integrins (Erdei et al. 2019 (27), modified). The activation of β₂ integrins is conformationally regulated by the inside-out signalling, which induces the separation of the α and β cytoplasmic tails. The 'switchblade-like' conformational change results in the active, extended state of the integrins, in which they are able to bind their ligands and fulfil their various functions.

Until now, several key proteins were identified to be involved in the regulation of integrin activation. Talins and kindlins activate integrins synergistically by binding to the intercellular tail of the β subunit (33,34), while filaminA has a negative regulatory effect (35). The integrin-linked kinases (36) and focal adhesion kinase (FAK) (37) enzymes can also affect the process of integrin activation. Migfilin regulates integrin activation by blocking the integrin-binding region of filamins (38). The small GTPase Rap1 (Ras-proximate-1) and its two effectors RapL (Rap-ligand) and RIAM (Rap1-interacting adaptor molecule) have also been found to participate in the talin dependent inside-out activation (39,40), from which RapL interacts with the α subunit, while RIAM stimulates the binding of talin to the β-tail (41,42). While our knowledge is limited about the intracellular signalling partners of CD11b and CD11c chains, it is postulated, that the activation kinetics of the distinct β₂ integrins can be different due to the different signalling pathways mediated by the distinct alpha cytoplasmic domains (43).

Beside the conformational changes, trafficking of integrins is also a dynamic process that can regulate receptor functions. Integrins are found to recycle rapidly between the cell membrane and the endosomal network (44), and this endocytosis can be mediated via different

routes, dependent or independent of clathrin (45). Rab proteins are also known to be involved in the process (46), either via a *short, Rab4-dependent pathway*, when the integrins return quickly to the cell surface from early endosomes (44,47) or by using the *Rab11-dependent long loop*, where integrins enter the perinuclear recycling compartment before they reappear in the plasma membrane (47,48). The membrane proximal sequence, required for recycling was also detected on the cytoplasmic tail of CD18 (49), and the β_2 integrin lymphocyte function-associated antigen 1 (LFA-1) was shown to be trafficked via a clathrin independent and Rab11-dependent pathway (50). The finding, that recycling of CR3 (CD11b/CD18) and LFA-1 (CD11a/CD18) is regulated differently however, suggests functional variances of β_2 integrins defined by their distinct α chains (44).

1.2.2. Outside-in signalling

After the integrins reach their active state on the cell surface and bind their ligands, they initiate the so-called outside-in signalling, which triggers the diverse functions mediated by these receptors. This intracellular cascade gives rise to the recruitment of numerous structural proteins, such as vinculin, paxillin and actin as well as signalling molecules including p130Cas, FAK and Rho (Ras homolog) GTPases. As a result, the rearrangement of the actin cytoskeleton takes place, and adhesive structures will be generated, which are involved in numerous functions including phagocytosis, adhesion or migration (27,51).

1.2.3. Differences between the two receptors

CR3 and CR4 contain identical β chain, while their α chain, namely CD11b and CD11c are closely related. The extracellular region of these latter two molecules contains the ligand binding domain, which is very similar, showing 87% homology (52). This explains the overlapping ligand recognition of the two receptors, however, it is interesting, that they bind iC3b at distinct sites (53). Due to their shared ligand specificity these receptors were generally thought to have similar function. At the same time, from an evolutionary point of view it does not seem economical to dedicate two different receptors to identical functions by the same cell (54). Moreover, in contrast to the extracellular region, the cytoplasmic tail of CD11b and CD11c has a highly different structure. This domain of CD11b has only 56% homology with the corresponding part of CD11c and is less than two-third of its size. Since this part of the molecule is involved in association with signalling and actin-binding proteins (43,55), the

difference between the cytoplasmic tail of CD11b and CD11c results in the different functional activity of CR3 and CR4. The distinct signalling properties together with the simultaneous presence of these receptors on leukocytes suggest a separation of functions.

1.3. Limitations of studies carried out using mice or cell lines

It is important to point out that the expression pattern of these receptors is fundamentally different in mice and men. In mice, CR3 is present on all myeloid cells, while CR4 is expressed in high numbers only on dendritic cells (DCs), insomuch that CD11c is often used as a marker to identify mouse DCs. In contrast to mice, in humans CD11c is simultaneously expressed next to CD11b on a wide variety of leukocytes (27,54,56,57). Namely, on monocytes, macrophages, dendritic cells, neutrophil granulocytes, and it has also been detected on certain populations of lymphoid cells as well (1,27,57,58)(**Table 1**).

	Human		Mouse	
	CR3	CR4	CR3	CR4
Monocyte	+	+	+	+/-
Macrophage	+++	+++	+	+/-
Dendritic cell	+++	+++	+/-	+++
Neutrophil	+	+	+	+/-
NK cell	++	+/-	+/-	+/-
T cell	+/-*	+/-*	+/-*	+/-*
B cell	+/-*	+/-*	+/-	+/-

+/- appears on certain subpopulations
* appears on activated and leukemic cells

Table 1. Expression of CR3 and CR4 on human and mouse cells (Erdei et al, 2019 (27))

Because the expression pattern of CR3 and CR4 is different in mice, the function of these receptors can be easily analysed separately on murine cells. In contrast to this, studying their individual role is way more challenging in humans due to their overlapping expression and the competition between the ligands for CR3 and CR4. Considering these facts, it can be easily accepted that data obtained in mouse studies cannot be simply translated to human system, but has to be handled with great caution. In line with this consideration, some differences are already known between the two species regarding the characteristics of CR3 and CR4. For example, mouse DCs down-regulate the cell surface expression of CD11c upon activation,

which is in sharp contrast to the behaviour of human DCs (59,60). Regarding the expression of CD11b on murine B cells it was demonstrated, that CD11b$^-$ B1 cells are the progenitors of CD11b$^+$ B1 cells (61), while human CD11b$^-$ B1 cells cannot be induced to express CD11b (62).

Another commonly applied experimental approach is the use of cell lines, expressing one of these receptors only, or both of them. With this method the natural variety between the donors can be excluded, which can be fairly high even among healthy donors, but is even higher among diseased people. A great benefit of using a homogenous cell line is that the role of the receptors can be studied individually on distinct cell types. However, it is important to emphasize, that cell line studies can only give information about the *possible* role of the receptors, but they do not prove that the receptors can *actually* carry out the found functions when expressed on primary cells.

Experiments carried out with the help of cell lines can be very useful in many cases, however, those methods have some serious disadvantages, especially when they are used to study the characteristics of a malignant disease. Ideally, a cell line established from a patient's sample has similar features to the founder patient's cells. However, even in this scenario, the cell line corresponds to the characteristics only of that particular patient's cells, which are not representative for the disease in general, due to the mentioned variability between the different patients. In a worse case, the cell line does not even correspond to the original features of the particular malignancy, in which scenario using them could even lead to fundamentally false conclusions (63,64). These problems can only be excluded for sure by using primary cells. For these reasons, in this thesis the presented new results as well as the cited literature focuses primarily on data obtained using cells isolated from human blood and tonsil.

1.4. Expression and role of CR3 and CR4 on myeloid cells

Both CR3 and CR4 are expressed on all human myeloid cells (27,54,57,65), although the extent of their appearance varies between cell types. Previously our research group assessed the number of these receptors on human monocytes, macrophages, dendritic cells and neutrophils. Comparing the relative number of CR3 and CR4 on monocytes and neutrophils, a significantly higher number of CR3 was found, while on macrophages and dendritic cells the CD11b:CD11c ratio is close to 1:1 (27,54) (**Table 2**). These differences may contribute to the functional diversity observed between these cell types, mediated by β_2 integrins.

	Monocyte	Macrophage	Dendritic cell	Neutrophil
CR3 (CD11b/CD18)	50 ± 8	310 ± 62	247 ± 21	47 ± 6
CR4 (CD11c/CD18)	7 ± 3	185 ± 40	204 ± 25	3,5 ± 2
Ratio of CD11b and CD11c	7,1	1,7	1,2	13,4

Table 2. The average number of CR3 and CR4 expressed by normal human myeloid cells (receptor number x10^3/cell) (Erdei et al., 2019 (27))

The best known function of CR3 and CR4 on human myeloid cells is phagocytosis. Both receptors are able to exert this function, as demonstrated by transfection of the non-phagocytic CHO cell line by CR3 and CR4 (66). Nonetheless, the actual contribution of these receptors to phagocytosis can be different among the various cell types, depending on the level of expression and also due to their most probably different signalling capacities. In line with the fact that on neutrophils and monocytes higher number of CD11b is expressed, CR3 was found to have a dominant role in the phagocytosis of these cell types. In the case of neutrophils only CR3 but not CR4 was found to mediate the phagocytosis of *Francisella tularensis* (67) and *Staphylococcus aureus* (68), which receptor also contributes to the phagocytosis of *Salmonella enterica* (69) and *Mycobacterium kansasii* (70). Similarly to that found on neutrophils, no significant contribution of CR4 was found on monocytes to the CR3 mediated phagocytosis of *Mycobacterium tuberculosis* (71) and *Staphylococcus aureus* (68). Further studies demonstrated also the dominant role of CR3 over CR4 on monocytes in the uptake of *Borrelia burgdorferi* (72), *Mycobacterium leprae* (73) and *Plasmodium falciparum* infected erythrocytes (74).

At the same time, macrophages and dendritic cells express similar amount of CR4 as CR3. Accordingly, in contrast to neutrophils and monocytes, where only CR3 was proven to be involved in phagocytosis, in case of macrophages both CR3 and CR4 mediate the binding and/or phagocytosis of *Cryprococcus neoformans* (75), *Francisella tularensis* (67) and *Mycobacterium leprae* (76). Downregulation of CR3 and CR4 in macrophages also hampered their phagocytic capacity for *Candida albicans* (77). The role of CR4 was even found to be dominant in the uptake of *Mycobacterium tuberculosis* by alveolar macrophages (71). In the case of dendritic cells both CR3 and CR4 were found to mediate side by side the phagocytosis of the opsonized, live *Francisella tularensis* (78).

However, most of these studies do not clearly differentiate between the uptake and digestion of the pathogens. For that reason, we have previously analysed these two sequential

processes separately. Using *Staphylococcus aureus* labelled with pHrodo Green (which is a pH sensitive dye that becomes highly fluorescent only in the acidic milieu of phagolysosomes), we proved the dominant role of CR3 in the digestion of iC3b opsonized *S. aureus* in the case of both neutrophils, monocytes, macrophages and dendritic cells (68,79). These data suggest that even where CD11c is expressed in a similar level as CD11b, CR3 dominates in the phagocytosis, while CR4 may have a more important role in other functions of the cells.

The involvement of CR3 and CR4 is also well known in cell adhesion. For example, dendritic cells have been shown to adhere to fibrinogen and secrete cytokines in a β_2 integrin-dependent manner (80). While only CR3 was found to be involved in the adhesion of phorbol myristate acetate (PMA) induced granulocytes (81,82), activated monocytes cultured in the presence of normal serum were shown to use both CR3 and CR4 for adhesion (83). CR4 was also found to be involved in the spreading of DCs and monocytes (54,84,85). Our research group proved that CR4 is the main receptor in the adhesion to fibrinogen on macrophages, dendritic cells and monocytes under physiological conditions (54), while under inflammatory conditions both CR3 and CR4 participate in the adhesion of macrophages and dendritic cells (60).

Moreover, these receptors have been shown to be equally important in podosome formation. Podosomes are specific adhesive structures of monocytic cells used for cell migration. These structures are generated and remodelled rapidly during migration on the contact surface of adherent cells (86). They consist of an F-actin core surrounded by an adhesion ring (87–89). Both CR3 and CR4 fulfil important function in the podosome formation (90,91), as they are located in the adhesion ring, as shown on macrophages and dendritic cells adhered to fibrinogen (68). Thereupon both receptors contribute to the migration of myeloid cells, as shown in monocytes, neutrophil granulocytes, macrophages and dendritic cells as well (43,60,92). The revealed functions of CR3 and CR4 on human myeloid cells are summarized on **Figure 4**.
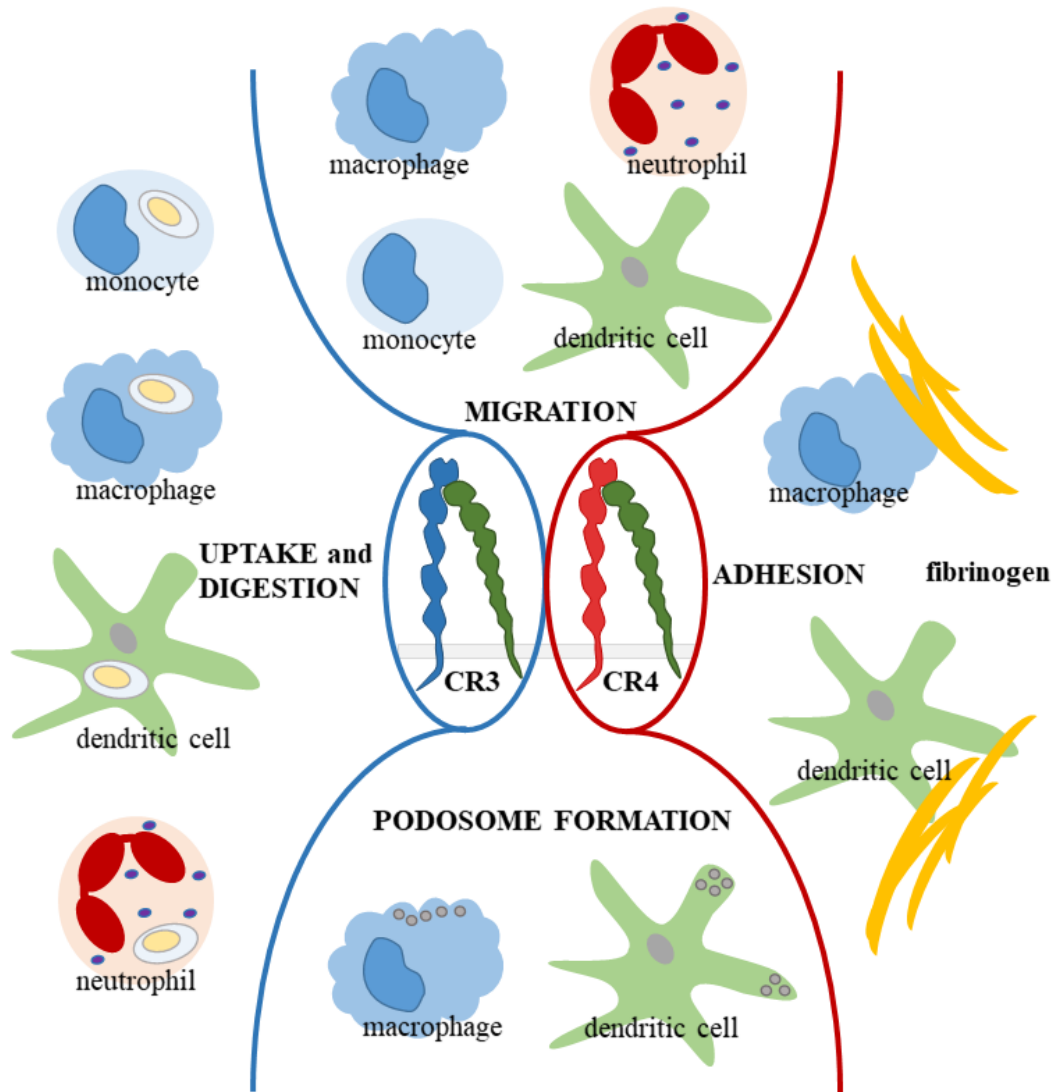
Figure 4. CR3 and CR4 mediated functions of human myeloid cells (Erdei et al. 2019 (27), modified). The best known function of CR3 and CR4 on human myeloid cells is phagocytosis. While both receptors are able to exert this function, we proved the dominant role for CR3 on both neutrophils, monocytes, macrophages and dendritic cells. In contrast, CR4 was found to be the main receptor in the adhesion to fibrinogen. Moreover, our group demonstrated on macrophages and dendritic cells that these receptors are equally important in podosome formation. Podosomes are specific adhesive structures of monocytic cells used for cell migration, thereupon both receptors contribute to the migration of myeloid cells, as shown in monocytes, neutrophil granulocytes, macrophages and dendritic cells as well.

1.5. Expression and role of CR3 and CR4 on lymphoid cells

1.5.1. NK cells

While CR3 is known to be expressed by natural killer (NK) cells, CR4 appears only on a subset of these lymphocytes (93–95). CR3 is present in preformed state inside these cells, and can be transferred rapidly to the cell surface following activation e.g. after phorbol ester treatment (93,96). CR3 was shown to promote the interaction of complement-opsonized target cells with the lytic effector NK cells, and thereby it contributes to the complement mediated enhancement of cytotoxicity (97). Regarding CR4, a CD11c$^+$ subset of NK cells was characterized recently with increased tumour cell cytotoxicity, interferon-γ (IFN-γ) producing capacity, and γδ T cell proliferation inducing property, however the exact function of CR4 in these functions has not been clarified yet (98).

1.5.2. T cells

CR3 is expressed by around 10% of human T cells. These CD11b$^+$ T lymphocytes belong mainly to the CD8$^+$ and CD56$^+$ population, and similarly to NK cells, contain preformed CR3, which is transferred quickly to the cell surface after PMA treatment (93). Longer period of activation yields CR3 expression in up to 28% of T cells, when CD4$^+$ T cells also acquire CD11b (96). In contrast to CR3, CR4 is not expressed by peripheral T lymphocytes, but can be detected on several cytotoxic T lymphocyte clones (57,99).

Regarding its role, CR3 was shown to contribute to the inhibition of anti-CD3-induced T cell proliferation and interleukin-2 (IL-2) release (96) and also to participate in the recruitment of CD8$^+$ T cells to sites of inflammation (100,101). However, while CD11b$^+$ T cells were shown to belong to subsets with killer properties (93), the exact role of CR3 in T cell cytotoxicity has not been clarified so far. Regarding CR4, it has been found, that CD11c expression correlates with high migratory properties and intense IFN-γ secretion of T lymphocytes (102). Moreover, CR4 was shown to contribute directly to the cytotoxicity of CD8$^+$ T cell clones (99).

1.5.3. B cells

Similarly to other lymphoid cell types, our knowledge about the expression and function of CR3 and CR4 on human B lymphocytes is limited and not always concordant. Nonetheless, the available information is discussed in the following paragraphs.

1.5.3.1. Expression and role of CR3 on B cells

Regarding the expression and role of CR3 on human B lymphocytes, various studies with often contradictory result can be found. According to Muto et al., around 20% of blood B lymphocytes are positive for CR3 (93), while Kawai et al. found that approximately 34% of B cells, belonging mostly to the CD27$^+$ memory pool, express CD11b. The expressed CR3 contributes to the high migratory potential of the B lymphocytes, as shown in a transwell assay, where CD11b blocking antibody reduced significantly the number of migrating B cells (5). Epigallocatechin gallate was also shown to exert its anti-inflammatory and anti-allergic effects by inhibiting the CR3 mediated adhesion and migration of blood B cells (103).

Griffin and colleagues found that approximately 10-13% of human B1 cells are CD11b$^+$, and these cells also express CR4. CD11b$^+$ B1 cells are either negative for CD5 or express only very low levels of it, in contrast to the typical CD11b$^-$ CD5$^+$ B1 B cells. Importantly, it has been shown that the CR3$^+$ B1 cells are not descendants of the CD11b$^-$ B1 cells, as both subsets retain their distinct CR3 expression levels when separately cultured, and stimulation of sorted CD11b$^-$ B1 cells did not give rise to CD11b$^+$ B lymphocytes. Moreover, it was also demonstrated, that CD11b$^+$ and CD11b$^-$ B1 cells fulfil different roles. Namely, CD11b$^+$ B1 cells stimulate allogeneic CD4$^+$ T cell expansion more efficiently, while the CD11b$^-$ population spontaneously secrete much more IgM than CD11b$^+$ B1 cells (62). Interestingly, while CD11b$^+$ B1 cells express elevated levels of the costimulatory molecule CD86, and thus drive T-cell proliferation, they are also found to secret IL-10 and suppress CD3-mediated T-cell activation. Thereby these cells are able to stimulate T-cell proliferation and to modulate T-cell activation as well. Due to their capacity of orchestrating aspects of immune responsiveness Griffin and colleagues termed these CD11b$^+$ B1 cells "orchestrator B1 cells" (104). Interestingly, the derailment of this regulation seems to contribute to the pathomechanism of systemic lupus erythematosus (SLE), as the frequency of these CD11b$^+$ B1 cells is elevated in lupus patients, where they express more CD86 and have increased T cell–stimulating activity (62). On the other hand, during self-protective immune reactions, Passos et al. showed that CD11b$^+$ B1 B cells can be activated by

Trypanosoma cruzi-derived protein-enriched fractions, and this activation is associated with a beneficial clinical status in Chagas disease (105). However, the direct contribution of CD11b to the observed functions was not investigated in these studies. Moreover, according to the analysis of Reynaud and Weill, it seems also possible, that the presence of these CD11b (and also CD14 and CD11c) bearing B1 cells is only a cell sorting artefact, and this subset represents a monocyte lineage that were sorted by unspecific (possibly Fc-mediated) staining as $CD19^+CD27^+$ cells (106). In their response, Griffin et al. demonstrated clearly, that $CD11b^+$ B1 cells are indeed B cells, on the basis of immunoglobulin gene rearrangement, surface immunoglobulin- and B cell receptor expression, cell morphology, and the presence of other characteristically B cell surface antigens as well (107). However, this debate is of particular interest, especially since in other studies human B lymphocytes were characterized as $CD11b^-$ cells (1,12).

Namely, Postigo et al. found that neither resting nor activated B cells of tonsils or blood express CD11b (1), which finding was strengthened lately by Uzonyi et al (12). However, it is important to point out that Griffin et al. demonstrated CD11b expression on only less than 1 % of total B lymphocytes (62), thus this very small amount of $CD11b^+$ B1 cells might easily escape the scope of the latter studies.

1.5.3.2. Expression and role of CR4 on B cells

Similarly to the literature of CR3 expression on B lymphocytes, the available data regarding the expression of CR4 on human B cells are also inconsistent. For example Wormsley and colleagues demonstrated CD11c expression on around 20% of blood B lymphocytes (3), while Postigo et al. detected no significant expression of CR4 on resting tonsillar or blood B cells. However, most of them became $CD11c^+$ after a three day long PMA stimulation (1). In 2011, $CD11c^+$ B cells were identified as $CD21^-$ and $CD19^{high}$ cells, and were further characterized as IgD^-, IgM^-, IgG^+, $CD38^{low}$, $CD5^{high}$, $CD80^{high}$, $CD86^{high}$, $CD20^{high}$, $CD23^-$, $CD27^{high}$, so basically as switched memory B cells (4). In a more recent study, Golinski et al. also described that the majority of CR4 bearing B lymphocytes belong to the switched memory $(CD27^+IgD^-)$ subset, however, $CD11c^+$ cells could also be detected among the unswitched $(CD27^+IgD^+)$ naïve $(CD27^-IgD^+)$ and double negative $(CD27^-IgD^-)$ B cells. The fact that CD11c was found to be expressed in each subpopulation suggests a role for CR4 in all steps of B cell development (6).

In their studies, Rubtsov et al. characterized the CD11c$^+$ population of B cells as "Age Associated B Cells" (ABCs) as they were detected in smaller numbers in the blood of healthy donors but were found to be expanded in rheumatoid arthritis and scleroderma patients, especially in older women. They showed in mice, that the accumulation of ABCs is driven by toll-like receptor 7 (TLR7), moreover, they seem to be involved in the development of autoimmunity by autoantibody production (4). The TLR7-induced accumulation of these CD11c$^+$ cells could explain their expansion in women, as TLR7 is coded by an X-linked gene, and thus it is overexpressed in females (108). The explanation of this phenomenon is that the inactivation of the females' extra X chromosome is not complete and only about 85% of genes are inactivated, while the rest of the encoded genes escape inactivation in humans' second X chromosome. This leads to the overexpression of some X-linked genes. That affects not only TLR7, but other immune-related factors as well, including CD40L, CXCR3, OGT, FOXP3, TLR8, IL2RG, BTK, and IL9R. Accordingly, more than 75% of autoimmune patients are women overall. The importance of X-linked gene dosage in the development of autoimmune diseases is strengthened by the fact that males with Klinefelter's syndrome (having an XXY genotype) have a similar risk for the development of lupus as the general female population, that is higher than in normal (XY genotype) male population (108).

However, the TLR7-driven accumulation of the ABCs was only shown in a murine model. It was also demonstrated, that while SLE patients have elevated expression levels of TLR7, the number of ABCs were not elevated in lupus patients, who came mostly from the younger generation (4). Moreover, Golinski and colleagues showed that the number of CD11c$^+$ B lymphocytes is unrelated to gender, and correlates only with the age of the healthy donors, varying between 3 to 55 percentages. Moreover, according to their results, the expression of CR4 is upregulated only upon B cell receptor (BCR) mediated activation but not through TLR9 or TLR7 (6). These findings might suggest a differential regulation of CR4 expression in B cells of men and mice, but the contradictory results could also be ascribed to the different methods used. In the experimental conditions applied by Golinski et al. TLR9 and TLR7 stimulation was tested to induce CD11c expression on CD11c$^-$ cells, which did not occur. However, this finding does not contradict to the possible effect of TLR stimuli to drive the accumulation of CD11c$^+$ cells by inducing their proliferation and not by the transition of CD11c$^-$ cells. Meanwhile, the gender-related nature of some autoimmunity-related factors can be explained not only by the X-linked overexpression of TLR7, but by a number of reasons including some sex-hormone based effects as well (109).

While the expression of CD11c on B lymphocytes have been studied by numerous groups, as mentioned previously, the function of the expressed CR4 on B cells was not clarified up to now. For instance, Golinski et al. demonstrated recently that CD11c$^+$ B cells differentiate into antibody-secreting cells upon activation, but the exact role of CR4 in this process has not been addressed in this study (6). Until now, CR4 was only demonstrated to be involved in the adhesion and proliferation by Postigo et al., who revealed that the ligation of CD11c with receptor specific antibodies triggers proliferation and blocks attachment to fibrinogen of PMA stimulated B cells (1). However, it is important to point out, that PMA is a non-physiological stimulator of the cells having a strong effect on integrins themselves (110), thus the measured function of CR4 on PMA stimulated cells may not correspond to the physiological role mediated by CR4 on B cells.

1.6. Chronic lymphocytic leukaemia – the relevance of CR3 and CR4 in its pathology

Integrins and other cell adhesion molecules have been shown to have an important role in the motility and trafficking of B cell lymphomas where the anatomical distribution of the malignant cells is related to their adhesion receptor expression profile (111). Among these adhesion receptors, β_2 integrins are also expressed by various B cell lymphomas, such as by diffuse large B cell lymphoma, mantle cell lymphoma, Hodgkin's lymphoma, CLL or Burkitt's lymphoma (111). Out of the detected β_2 integrins CR3 and CR4 have been shown to be expressed in Hodgkin's lymphoma (112), hairy cell leukaemia (9) and in chronic lymphocytic leukaemia (7–14).

Among these CR3 and CR4 bearing B cell malignancies, CLL is the most common type of leukaemia in the western world with incidence of 4.1/100 000, from which the majority of the patients being elderly (113). Characteristically, CLL B cells have a clonal memory phenotype and accumulate and crowd out healthy cells from the blood, lymph nodes, spleen, bone marrow and other lymphatic tissues (114,115). They proliferate typically in the bone marrow and lymph nodes, where they gain anti-apoptotic and pro-survival signals from the stromal microenvironment (116). Moreover, this stromal microenvironment can contribute to the chemoresistance as well, and accordingly, residual cells remain often in the bone marrow and lymph nodes, even when therapies are effective at killing CLL cells in the blood (116,117). These characteristics of CLL pathomechanism explain, why every factor, that affects the

dissemination of the malignant B cells, can potentially influence the outcome of the disease. For this reason, the expression profile of cell-adhesion and migration molecules (such as CR3 and CR4), and their correlation with disease progression is an extensively studied field, even if numerous questions are still waiting to be answered.

Various proportions of CD11c$^+$ B cells were detected previously in the blood of CLL patients, namely 21% (10), 26% (7), 27% (8), 40% (11), 49% (9) or even 89% (14). The prevalence of CD11b varied between 20% (8) and 66% (10). Despite the highly varying appearance of CR3 and CR4 in the patients, several groups demonstrated that their expression correlates with the progression of the disease (7,8,10,11). Since the profile of the adhesion molecules expressed by B cell lymphomas can influence their anatomical distribution (111), it can be assumed that the presence of CR3 and CR4 might lead to the elevated adhesive and migratory behaviour of leukemic B cells to retain in the bone marrow or reach different organs effectively. This hypothesis is further strengthened by the fact that the presence of either CR3 or CR4 was found to be an unfavourable prognostic factor in CLL as the expression of both β_2 integrins correlates with the pattern of bone marrow infiltration (7).

However, the exact function of CR3 and CR4 on CLL B cells is still not clarified. For this reason, we set out to unravel the role of these integrins in chronic lymphocytic leukaemia. In 2017 our research group studied the expression and role of CD11b and CD11c of the B cells of two CLL patients, who were characterized by a peculiar immune-phenotype containing both CD5$^+$ and CD5$^-$ B cell subsets. Both CR3 and CR4 could be detected on CD5$^+$ as well as on CD5$^-$ CLL cells, albeit to different extents. The receptors were found to be involved in the spreading of CpG-activated CLL B cells on fibrinogen, as this function could be partially blocked by monoclonal antibodies specific for CD11b or CD11c (**Figure 5**)(12).
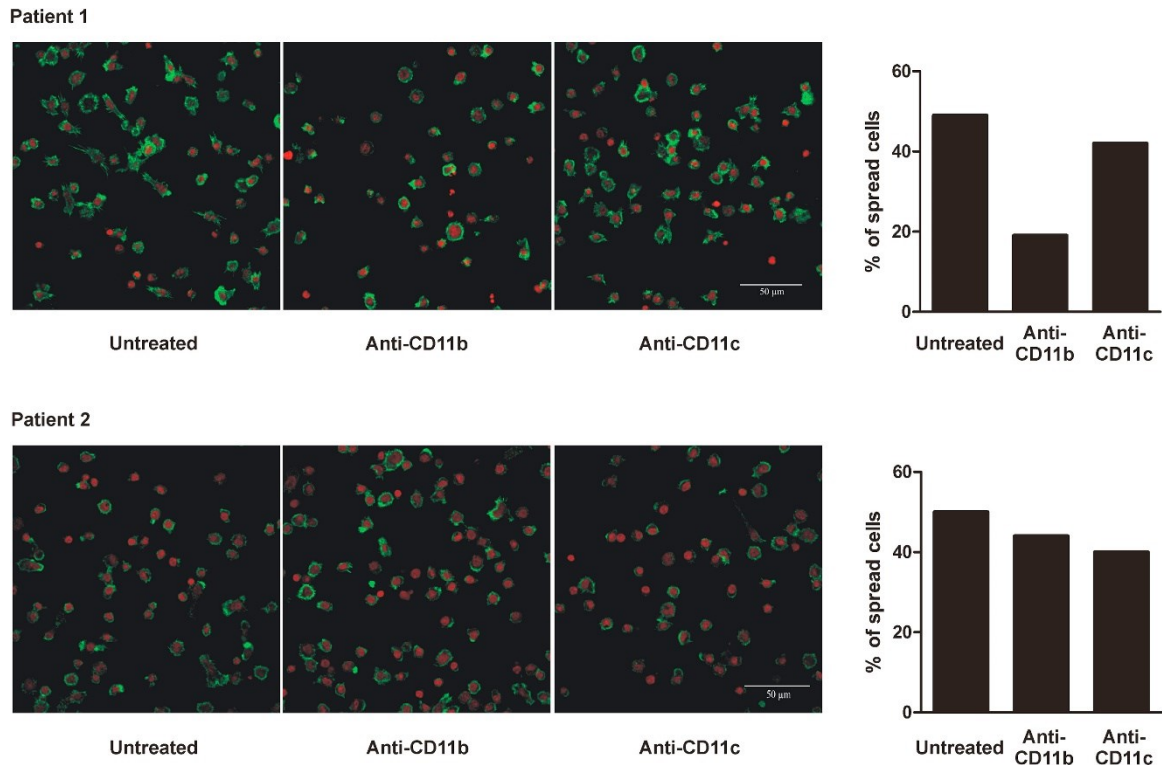
Figure 5. CR3 and CR4 are involved in the spreading of CpG-activated CLL B cells on fibrinogen. Data of two patients characterized by a peculiar immune-phenotype containing both CD5$^+$ and CD5$^-$ cells are shown. The figure is taken from the paper of Uzonyi et al. (12).

Beside the function of CR3 and CR4, it was found that activation via TLR9 results in the proliferation of both CLL B cell subsets, however with a less pronounced effect on the CD5$^+$ population. Activation by CpG also led to a strong IL-10 production by the B cells of both patients, while the simultaneous trigger via BCR and TLR9 resulted either in a synergistic enhancement of proliferation and IL-10 production or inhibited the same processes exerted by the patients' B cells (12). However, this study focused on the special characteristics of these two peculiar patients, who were characterized with both CD5-positive and CD5-negative CLL B cell subsets. In contrast to them, in the case of the majority of CLL patients clonal memory B cells are accumulating with an invariant immunophenotype (114,115). For this reason, it is necessary to investigate additional CLL patients with conventional phenotype, focusing especially on the various functions of CR3 and CR4, to gain further insight into the role of these receptors in the pathomechanism of chronic lymphocytic leukaemia.

2. Aims

While the expression and role of CR3 and CR4 is well studied on myeloid cells, our knowledge about their function on human B lymphocytes is limited. The purpose of the research presented in this thesis has been to gain a deeper insight about the expression and role of these receptors on primary human B cells under physiological conditions to model their *in vivo* functions in health and disease.

Our aims were:

1. To characterize the expression of CR3 and CR4 on resting and activated B cells of healthy donors, as well as the kinetics of the expression and to identify the other characteristics of the receptor-bearing cells.

2. To reveal the role of the β_2 integrin type complement receptors on B lymphocytes of healthy donors in order to describe their function in normal immune response.

3. To investigate the expression of CR3 and CR4 on B cells of chronic lymphocytic leukaemia patients.

4. To reveal the role of the β_2 integrin type complement receptors on B cells of CLL patients in order to better understand their contribution to the pathomechanism of the disease.

3. Materials and methods

3.1. Isolation of B cells

3.1.1. Tonsillar B cells

Tonsils were obtained from children undergoing routine tonsillectomy in the Central Hospital of Southern Pest National Institute of Hematology and Infectious Diseases in Budapest, Hungary, in accordance with the Helsinki Declaration and was approved by the Ethics Committee of the Medical Research Council in Hungary (TUKEB), 52088/2015/EKU. After mechanical destruction of the tonsillar tissue, mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation (GE Healthcare, Chicago, IL, USA). T lymphocytes were rosetted by the addition of 2-aminoethylisothiouronium bromide (Sigma-Aldrich, St. Louis, MO, USA) treated sheep red blood cells, and B cells were isolated by a second centrifugation over Ficoll-Hypaque solution. For each experiment, high-density ("resting") B cells were used, separated on Percoll (Sigma-Aldrich, St. Louis, MO, USA) gradient. The purity of B cells was higher than 95% in each case, as measured by flow cytometry using FITC-conjugated mouse-anti-human CD19 antibody (clone LT19, ImmunoTools GmbH, Friesoythe, Germany). For the RQ-PCR measurements tonsillar B cell samples were further purified by sorting CD19 positive cells using a FACS Aria III instrument and the FACSDiva software, reaching a purity higher than 99%.

3.1.2. Peripheral blood B lymphocytes

Peripheral blood B lymphocytes were isolated from buffy coat obtained from healthy donors and provided by the Hungarian National Blood Transfusion Service. Informed consent was provided for the use of blood samples according to the Helsinki Declaration. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque gradient centrifugation and B cells were isolated by negative selection using the Pan B Cell Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany).

3.1.3. Malignant B cells

Blood samples of patients diagnosed with chronic lymphocytic leukaemia were obtained from the Department of Internal Medicine and Oncology of Semmelweis University with their clinical data summarized in **Table 3**. None of these patients required therapy at the time of the study, they were monitored using a watch and wait strategy. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Hungarian Medical Research Council Scientific and Research Committee (ETT TUKEB, permission number: 21655-1/2016/ EKU).

	age (years)	sex	Rai stage	IGHV status
Patient 1	72	female	I	mutated
Patient 2	73	male	I	not available
Patient 3	65	female	I	mutated
Patient 4	67	male	I	mutated
Patient 5	47	female	I	mutated
Patient 6	71	female	I-II	mutated
Patient 7	66	female	II	not available
Patient 8	71	male	I	unmutated

Table 3. Characteristics and clinical data of CLL Patients at the time of the study.

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation from patients' EDTA-treated venous blood. Patients' B cells were purified by negative selection using the Miltenyi B-CLL Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) achieving >97% purity, verified by CD19 expression. In some experiments BJAB, an African Epstein-Barr virus (EBV) negative Burkitt-like lymphoma cell line obtained from American Type Culture Collection (ATCC; U.S.A.) was used.

3.2. Culture conditions

Isolated tonsillar or blood B cells of healthy donors were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FCS (Thermo Scientific, Rockford, IL, USA) and 50 µg/ml gentamycin (Sigma-Aldrich) at 37ºC and 5% CO_2, and were activated using 5 µg/ml F(ab')$_2$ anti-human IgG/A/M (Jackson ImmunoResearch, Cambridgeshire, UK) and/or 0.5 µg/ml CpG-ODN 2006 (Sigma-Aldrich, St. Louis, MO, USA) for 3 days as indicated. Chronic lymphocytic leukemic B cells were cultured in the same medium containing 10% FCS

and 50 μg/ml gentamycin at 37ºC and 5% CO_2, in the presence of 50 ng/ml IL-2 (ImmunoTools GmbH, Friesoythe, Germany) and 5 μg/ml goat anti-human IgG/A/M F(ab')$_2$ antibody. Functional studies were carried out on the 3rd day of the culture.

3.3. Flow cytometry

Flow cytometry was carried out right after isolation and at day 3 of culturing. To measure CD11b expression on B cells of healthy donors mouse anti-CD11b antibody (clone ICRF44, Biolegend, San Diego, CA, USA) with Alexa 647 conjugated anti-mouse antibody (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) were used. To characterize CD11c positive B cells the following antibodies were used: PE-conjugated anti-CD11c (clone BU15, ImmunoTools GmbH, Friesoythe, Germany), APC-conjugated anti-CD27 (clone LT27, ImmunoTools) and biotin-conjugated anti-human IgD (BD Biosciences, San Jose, CA, USA) with Streptavidin, Alexa Fluor 488 conjugate (Invitrogen).

To verify that β$_2$ integrins on BCR-activated CD11c$^+$ B cells switched to their active state under the conditions of the functional assays, we used an Alexa Fluor 488 conjugated antibody specific to the active conformation of CD18 (clone m24, Biolegend, San Diego, CA, USA).

The absolute number of CD11c was determined by using Qifikit (Dako, Agilent, Santa Clara, CA, USA) according to the manufacturers' instructions. Briefly, cells were labelled with saturating concentration of mouse-anti-human CD11c antibody (clone BU15, ImmunoTools) and with FITC-conjugated F(ab)'$_2$ fragment goat anti-mouse secondary antibody (Jackson ImmunoResearch, Cambridgeshire, UK). Beads carrying defined amounts of mouse IgG were labelled with the same secondary antibody to specify the correlation between fluorescence intensity and the number of antibodies bound. The calibration curve established with the beads was used to determine the number of bound anti-CD11c antibodies on the surface of B lymphocytes.

The expression of CLL B cells was characterized using the following antibodies: anti-CD11b (clone ICRF44, Biolegend, San Diego, CA, USA), anti-CD11c (clone BU15, ImmunoTools GmbH, Friesoythe, Germany), anti-CD41a (clone HIP8, IgG1, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), anti-CD51/CD61 (clone 23C6, IgG1, Invitrogen) and anti-CD49e (clone SAM1, IgG2b, Invitrogen) with Alexa Fluor 647-conjugated goat anti-mouse antibodies (Thermo Fisher Scientific Inc.).

To rule out dead cells from the analyses we used 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Scientific, Rockford, IL, USA) or propidium iodide (Thermo) staining. Measurements were performed using a CytoFLEX cytometer (Beckman Coulter Life Sciences, Indianapolis, IN, USA) employing the CyteExpert software. Data were analysed using the CytExpert and Kaluza softwares.

3.4. RNA isolation and real-time quantitative PCR

B cells of healthy donors were collected immediately after sorting or following BCR mediated activation and total RNA was isolated using TRIzol Reagent (Invitrogen, Thermo Fisher Scientific) following the manufacturer's protocol. Hundred ng of the isolated RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA).

The real-time quantitative polymerase chain reaction (RQ-PCR) assay was performed with Quantstudio® 3 Real-Time PCR System (Thermo Fisher Scientific) and Quantstudio Design & Analysis Desktop Software version 1.4 (Thermo Fisher Scientific) was used to analyse the data after amplification. For amplification of CD11b and CD11c coding mRNA predesigned Taqman assays for integrin subunit α_M, *ITGAX* (Hs00174217_m1) and integrin subunit α_X, *ITGAM* (Hs01064805_m1) (Thermo Fisher) were used. RQ-PCR was performed in duplicates, for 40 cycles (95°C for 1 s, 60°C for 20 s), and the relative quantity of each mRNA was calculated applying the comparative C_T method using human β-glucuronidase *GUSB* (Hs99999908_m1, Thermo Fisher) endogenous control as reference gene.

3.5. Adhesion assay

Adherence of B cells of healthy donors and CLL patients was assessed on the 3rd day of the culture. We also measured the adhesive capacity of CD11c positive BJAB cells. Before and during the assay cells were incubated with Fc-receptor blocking reagent (Miltenyi Biotec) to avoid Fc-receptor mediated binding of the integrin-specific antibodies. For blocking the function of integrins, cells were treated for 30 minutes at 4 °C with 10 μg/ml of one of the following antibodies: anti-CD11b (clone ICRF44, Biolegend), anti-CD11c (clone BU15, ImmunoTools), anti-CD41a (clone HIP8, IgG1, Invitrogen), and anti-CD49e (clone SAM1, IgG2b, Invitrogen), as indicated. All of the used integrin specific antibodies were previously shown to block the function of the target molecule (5,118–120). As control, isotype matched

control antibodies were used (mouse IgG1, clone OKT9, Thermo Fisher Scientific and mouse IgG2b, clone MPC-11, Biolegend). To ensure that integrins recycled from the cytoplasm are also blocked, the antibodies were not washed out for the assay. Ninety-six-well CELLview cell culture dish with glass bottom (Greiner Bio-One, Kremsmünster, Austria) was coated with 10 µg/ml fibrinogen (Merck, Budapest, Hungary) for 1 h at 37°C. After washing with phosphate-buffered saline (PBS), free surfaces were blocked with 250 µg/ml synthetic copolymer poly (L-lysine)-graft-poly (ethylene glycol) (PLL-PEG, SuSoS AG, Dübendorf, Switzerland) for 1 h at 37 ºC. As negative control, we measured the number of adhered cells to PLL-PEG blocked surfaces in the absence of fibrinogen coat. After blocking the cells with antibodies as mentioned above, they were allowed to adhere to the fibrinogen-coated and/or PLL-PEG blocked surfaces for 1 h at 37 °C and 5% CO_2 in 100 µl of medium. After fixing with 2% paraformaldehyde (Sigma-Aldrich) for 10 minutes unbound cells were washed away with PBS and the adherent cells were stained with Draq5 (Biolegend) and phalloidin-Alexa488 (Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA) containing 0.1% Triton X-100 (Reanal, Budapest, Hungary). Images were taken by an Olympus IX81 laser scanning confocal microscope using the FluoView 500 software. Eight representative fields were scanned in two wells for each treatment and nuclei were counted using ImageJ software.

3.6. Migration assay

The measurement of migration was performed on B cells of healthy donors and CLL patients on the 3rd day of the culture. Before and during the assay cells were incubated with Fc-receptor blocking reagent (Miltenyi Biotec) to avoid Fc-receptor mediated binding of the specific antibodies. For blocking the function of integrins, cells were treated for 30 minutes at 4 °C with 10 µg/ml of anti-CD11b (clone ICRF44, Biolegend), anti-CD11c (clone BU15, ImmunoTools), or isotype matched control antibodies (mouse IgG1, clone OKT9, Thermo Fisher Scientific) as indicated. To ensure that integrins recycled from the cytoplasm are also blocked, the antibodies were not washed out for the assay. The migration assay has been performed using 24 well Transwell plates (polycarbonate membrane with 5.0 µm pore, Corning, NY, USA) towards 100 ng/ml stromal cell-derived factor-1 (SDF-1) (Thermo Fisher Scientific). Transwell membranes were coated with 100 µg/ml fibrinogen in PBS overnight at 37 °C and masked with 250 µg/ml PLL-PEG for 1 h at 37 ºC. Antibody-treated cells were added to the upper chamber of the transwell assay in 100 µl RPMI-1640 medium containing 10% FCS and 50 µg/ml gentamycin, while the lower chamber of the assay contained 100 ng/ml SDF-1 diluted in 600 µl of the same

medium. As negative control, we measured the number of migrated cells through PLL-PEG masked transwell membranes in the absence of fibrinogen coat and/or the chemoattractant SDF-1. After the cells were allowed to migrate for 4 h at 37 °C and 5% CO_2, 25 mM EDTA was added to the lower chamber and the upper chamber was removed. Transmigrated cells were collected from the lower chamber, and cell number was counted immediately using a CytoFLEX cytometer (Beckman Coulter Life Sciences).

3.7. Proliferation assay

Freshly isolated high-density B cells were seeded onto 96-well flat bottom culture plates precoated with 10 μg/ml fibrinogen and/or blocked with 250 μg/ml PLL-PEG where indicated, in triplicates, at 2×10^5 cells/well. Cells were stimulated with 5 μg/ml goat anti-human IgG/A/M F(ab')$_2$ (Jackson ImmunoResearch, Cambridgeshire, UK) for 3 days. After 48 hours cells were pulsed with 1 μCi/well ^{3}H-thymidine (NEN, Boston, MA, USA) for 18 hours. Incorporated radioactivity was measured with a Wallac 1409 liquid scintillation β counter (Wallac, Allerod, Denmark).

3.8. Statistics

We compared each treatment to the appropriate control sample, presented on the graphs as 100%. Statistical tests were performed with GraphPad Prism 6 software, with $p < 0.05$ considered significant.

4. Results

4.1. Expression of CR3 and CR4 on B cells of healthy donors

4.1.1. Activated human B lymphocytes express CR4 but not CR3

As it is described in detail in the introduction, numerous studies with often contradictory result can be found regarding the expression of CR3 and CR4 on human B lymphocytes. Several papers can be cited which show that human B cells express CR3 (2,5,93) or CR4 (4,6,121), as well as studies showing that they express neither of these receptors (1,12). To find out whether human B cells do or do not express these complement receptors we carried out a detailed investigation. We began our study by the measurement of the expression of CR3 and CR4 on freshly isolated and activated B cells of healthy donors by flow cytometry. As it was also suggested by different groups earlier, that the expression can be triggered by different stimuli (1,4,6), we decided to analyse this issue as well. Investigating the influence of activation on integrin expression we deemed important to apply physiologically relevant stimulations, thus we activated B cells via the BCR and TLR9. As peripheral lymphoid organs are the primary site for B cell activation, furthermore tonsils contain a wider range of various B cell populations than peripheral blood, we compared the expression of CD11b and CD11c on B cells of both sources.

As shown in Figure 6, on resting tonsillar B cells no CR3, and only a slight amount of CR4 was detected. After 3 days of BCR-stimulation with 5 μg/ml goat anti-human IgG/A/M F(ab')$_2$, up to 35% of the cells expressed CD11c. Activation with 0.5 μg/ml of CpG, the TLR9 agonist also induced CD11c expression in up to 21% of B cells, however, the average ratio of CD11c$^+$ cells among TLR9 stimulated tonsillar B lymphocytes was not significantly higher than that of the non-stimulated B cells. Interestingly, the simultaneous trigger induced a lower percentage of CD11c$^+$ B lymphocytes than the BCR-stimulus alone. In the case of blood-derived B cells we also found that BCR-stimulation was the strongest trigger to induce CD11c expression, while lower percentages of B lymphocytes were positive for CD11c after stimulation via TLR9 alone or via TLR9 and BCR combined. In contrast to CR4 however, none of the indicated stimuli induced CD11b expression on tonsillar or blood-derived B lymphocytes (**Figure 6**).
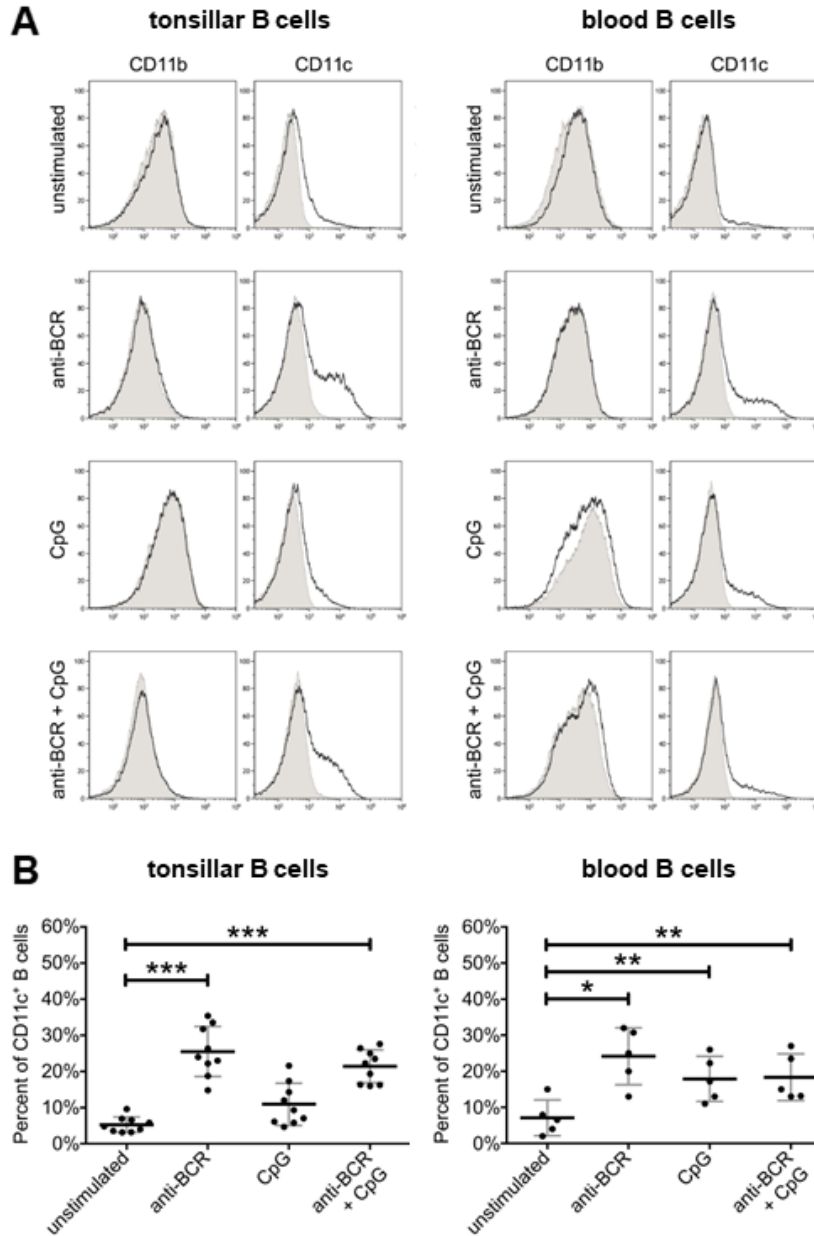
Figure 6. Expression of CD11b and CD11c on human B lymphocytes. CR3 and CR4 expression was measured by flow cytometry using unstimulated tonsillar and peripheral blood B lymphocytes, and cells after activation with 5 μg/ml F(ab')$_2$ anti-human IgG/A/M or 0.5 μg/ml CpG either separately or simultaneously. The purity of B cells was higher than 95% in each case, as measured by CD19 positivity. To rule out dead cells from the analyses we gated on cells which were negative for DAPI staining. Histograms show the results of one representative experiment (**A**), while the diagrams summarize the results (mean +/- SD) calculated from 9 independent experiments in the case of tonsillar, and 5 independent experiments in the case of blood-derived B lymphocytes. One-way ANOVA with Tukey's post-test was used (*=p < 0.05; **=p < 0.01; ***=p < 0.001). (**B**).

4.1.2. Kinetics of BCR-induced CR4 expression on B cells of healthy donors

While the kinetics of protein-expression is determined mostly by transcription and translation, in the case of β_2 integrins the intracellular trafficking is known to be a dynamic process as well. Since by flow cytometry we can detect CD11c molecules appearing on the cell surface only, it is also possible, that B cells contain preformed receptors which are transferred rapidly to the surface from intracellular pools following activation. This scenario had been shown e.g. for CR3 in the case of neutrophils, NK cells and T cells (93). For this reason, we set out to define the kinetics of CD11c expression after BCR-stimulation. To this end, we assessed CR4 expression at mRNA and protein level simultaneously on days 1, 2 and 3 after activation.

Flow cytometry measurements revealed that on tonsillar B lymphocytes the cell surface expression of CR4 increases on day two after activation and reaches its maximum on day 3 (**Figure 7A**). Our RQ-PCR analysis revealed that the amount of coding mRNA for CD11c (*ITGAX*) elevates after 24 hours of stimulation and reaches its maximum on the second day, i.e. one day before the maximum of the protein expression on the cell surface (**Figure 7B**). This finding implies that the CR4 molecules appearing on the B cell surface are newly synthesized. Verifying our cytometry data, no enhanced expression of the coding mRNA for CD11b (*ITGAM*) was found in the case of non-stimulated or stimulated B cell samples (data not shown).
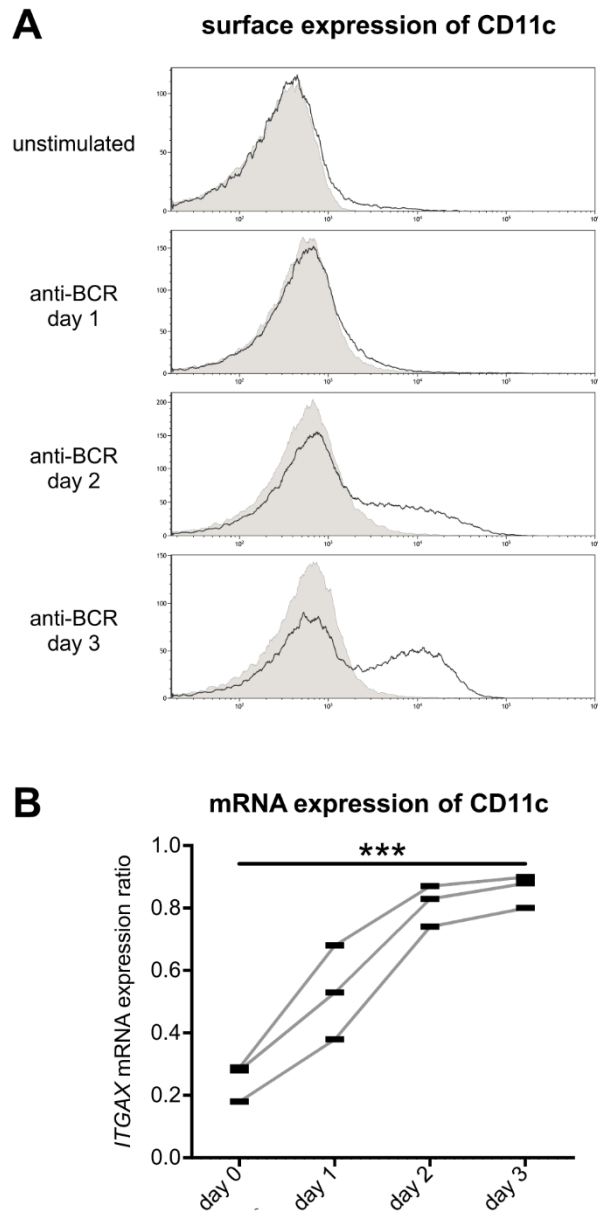
A surface expression of CD11c

unstimulated

anti-BCR day 1

anti-BCR day 2

anti-BCR day 3

B mRNA expression of CD11c

***

$ITGAX$ mRNA expression ratio

day 0 day 1 day 2 day 3

Figure 7. Kinetics of CR4 expression on BCR-activated tonsillar B lymphocytes. The surface expression of CD11c was measured by flow cytometry on resting tonsillar B cells and after 1, 2 or 3 days of activation using 5 µg/ml goat anti-human IgG/A/M F(ab')$_2$. Results of one representative of 3 independent experiments are shown (**A**). Values of RQ-PCR analysis represent expression ratios of the CD11c coding mRNA $ITGAX$ normalised to the expression level of the housekeeping gene $GUSB$ (3 independent experiments). We used one-way ANOVA with post-test for linear trend to test whether the values increase from left to right order (*=p < 0.05; **=p < 0.01; ***=p < 0.001) (**B**).

4.1.3. Number of CD11c molecules on the surface of activated human B cells

So far, the density of CR4 molecules on human B lymphocytes has not been determined, though this information is important to better understand the role of CD11c in various B cell functions. For this reason, we assessed the number of this β_2 integrin on day 3 after the BCR-stimulus. Using the Qifikit calibration beads, we revealed that on average 9500 CD11c molecules are present on the cell surface (**Figure 8**). This density is higher than that measured on monocytes (54) or neutrophil granulocytes (27) (**Table 2**), indicating a level of expression that is high enough to exert important functions of B lymphocytes.
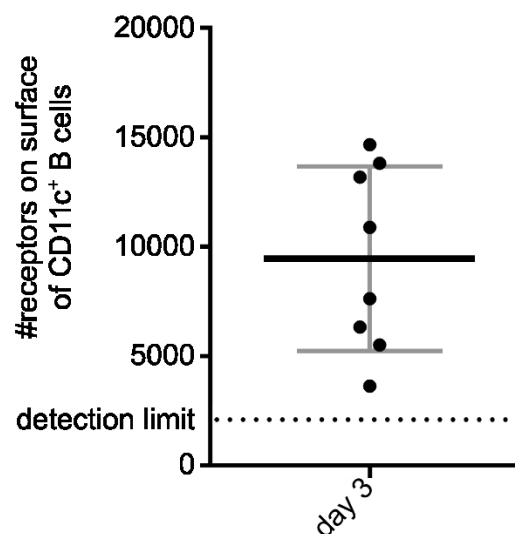

Figure 8. Number of CD11c molecules on the surface of activated human B cells. The number of CD11c was determined by flow cytometry using Qifikit calibration beads on tonsillar B cells 3 days after BCR-induced activation (mean +/- SD was calculated from 8 independent experiments).

4.1.4. β$_2$ integrins on BCR-activated CD11c$^+$ B cells are functionally active

As reviewed in the introduction, the activity of the integrins is conformationally regulated. Without activating signals, these receptors are in their inactive, bent conformation on the cell surface. Their activation is induced by inside-out signalling, depending on the actual state of the cell. Since integrins are able to bind their ligands and fulfil various functions only in their extended, active conformation, we tested whether β$_2$ integrins on BCR-activated CD11c$^+$ B cells are expressed in their functionally active state.

We labelled the cells using an Alexa Fluor 488 conjugated antibody specific to the active conformation of CD18 on ice (black) or at 37°C for 30 minutes (red). Staining by the isotype control antibody is shown in grey (**Figure 9**). While on ice β$_2$ integrins are in their inactive state, raising the temperature to 37°C itself was enough to turn them into their active conformation. Thus we confirmed that β$_2$ integrins are able to switch into their active state on BCR-activated CD11c$^+$ B cells.
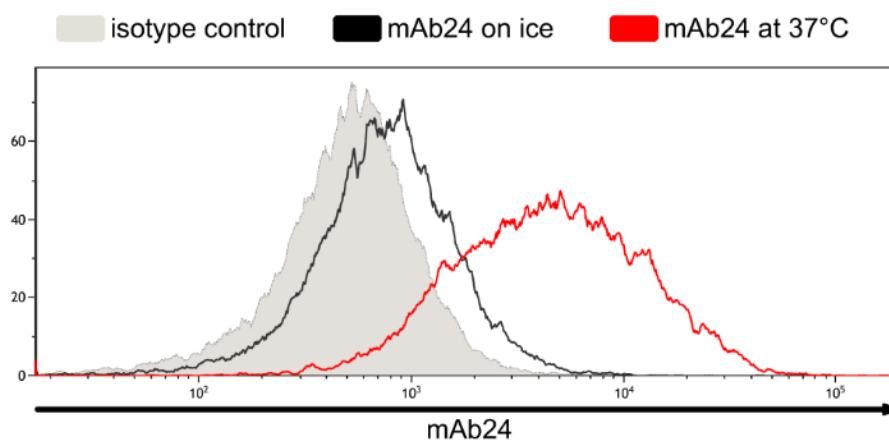
Figure 9. β$_2$ integrins are in active conformation on BCR-activated CD11c$^+$ B cells under the conditions of the functional assays. Cells were labelled with Alexa Fluor 488 conjugated antibody specific to the active state of CD18 in medium, on ice (black) or at 37°C for 30 minutes (red), or whit isotype control antibody (grey). While on ice β$_2$ integrins are in their inactive state, raising the temperature to 37°C itself was enough to turn them into their active conformation. Results of one representative of 3 independent experiments are shown.

4.1.5. Activation-induced CD11c expression of memory B cells occurs parallel with Ig class switching

Rubtsov et al. characterized blood derived CD11c$^+$ B lymphocytes as IgD$^-$, IgM$^-$, IgG$^+$, CD38^{low}, CD5^{high}, CD80^{high}, CD86^{high}, CD20^{high}, CD23$^-$, CD27^{high} B cells [4], that are basically switched memory B cells. In a more detailed study of their classification approximately 62% of CD11c$^+$ B cells were found to represent the switched (CD27$^+$IgD$^-$) and 16% the unswitched (CD27$^+$IgD$^{+)}$ memory B cell pool. At the same time, the frequency of CD11c$^+$ B cells was reduced in the naïve (CD27$^-$IgD$^+$) as well as in the double negative (CD27$^-$IgD$^-$) population. Yet, CD11c was detected in each subpopulation, suggesting a role for CR4 in all steps of B cell development [6]. Since the phenotype of tonsil derived CD11c$^+$ human B cells has not been defined so far, we set out to characterize this population. We also compared the distribution of CD11c$^+$ B lymphocytes in the population of unstimulated and BCR-activated B cells.

By triple-staining of unstimulated tonsillar B cells for CD27, IgD and CD11c we found that the vast majority of tonsillar CD11c$^+$ B cells also belong to the switched memory population (CD27$^+$IgD$^-$). Lower numbers of CD11c$^+$ B cells were found in the unswitched memory (CD27$^+$IgD$^+$) population and only a few CD11c$^+$ cells were detected among the double negative (CD27$^-$IgD$^-$) and naive (CD27$^-$IgD$^+$) B cells (**Figure 10A**). While blood and tonsils contain different subsets of B lymphocytes (e.g. blood lacks germinal center (GC) B cells), we found that the distribution of non-activated CD11c$^+$ B cells in tonsil and blood is very similar (**Figure 10B**).

However, when comparing the distribution of CD11c$^+$ B cells after 3 days of activation via the BCR, one can observe significant changes in the case of tonsillar B lymphocytes. While the frequency of switched memory (CD27$^+$IgD$^-$) as well as of double negative (CD27$^-$IgD$^-$) B cells increased significantly in the CD11c$^+$ B cell pool, the frequency of unswitched memory B cells and naïve B cells decreased (**Figure 10A**). Though BCR-activation induced the increase of CD11c expressing tonsillar as well as blood B cells (**Figure 6**), in the case of the latter no significant changes were observed regarding their distribution (**Figure 10B**).

Overall, our data show that while the vast majority of both tonsil and blood derived CD11c$^+$ B cells belong to the switched memory pool with or without BCR-stimulation, the ratio of switched memory B cells further increases among tonsillar CD11c$^+$ B cells after 3 days of activation. This suggests that the activation induced CD11c expression of tonsillar B cells occurs mostly in parallel with class switching.
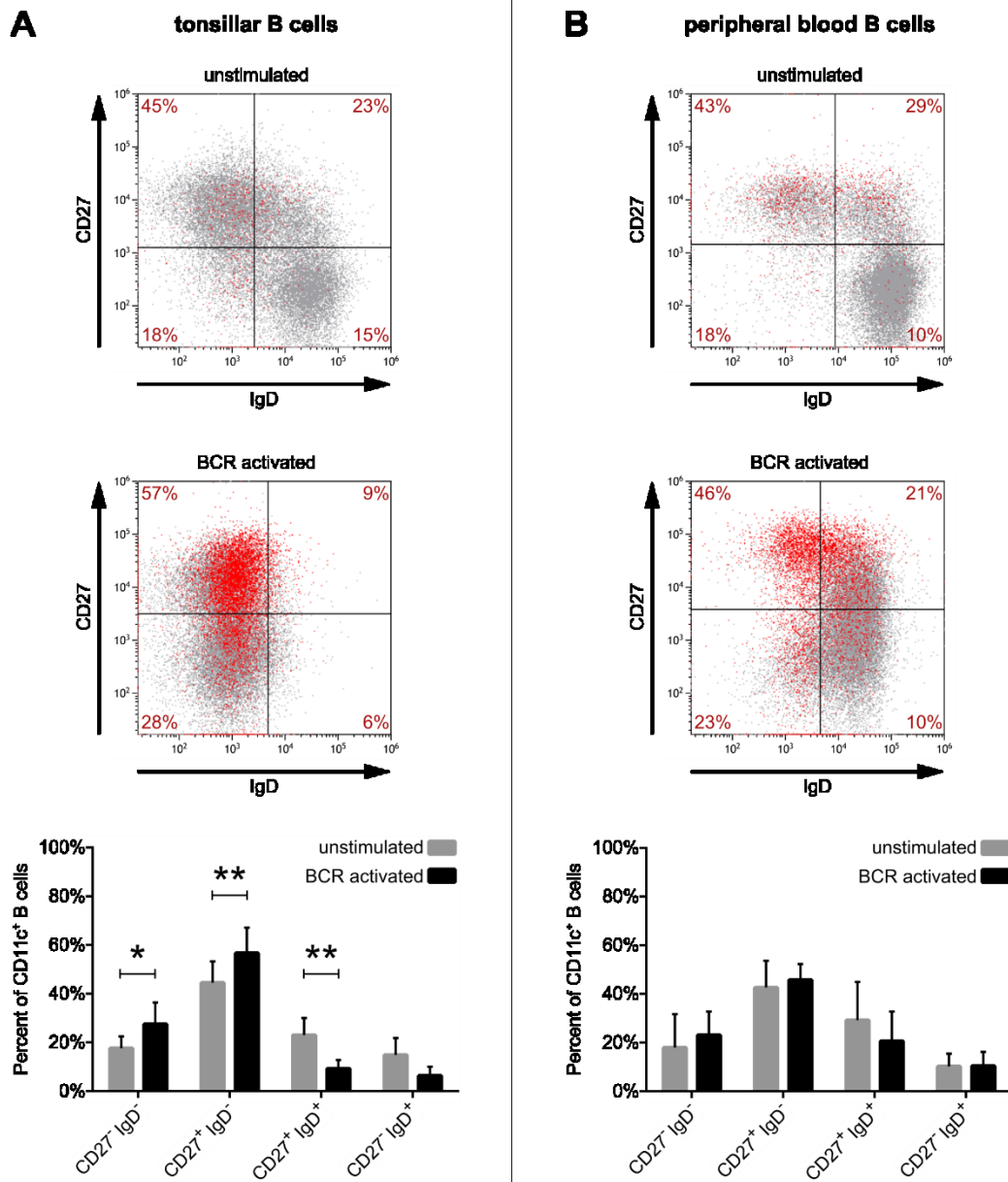
Figure 10. The phenotype of CD11c$^+$ human B cells derived from tonsil and blood.
Surface expression of CD27, IgD and CD11c was assessed by flow cytometry right after isolation (unstimulated) or after 3 days of activation with 5 μg/ml goat anti-human IgG/A/M F(ab')$_2$ (BCR-activated) on tonsillar (**A**) or blood-derived B cells (**B**). Dot-plots show the results of one representative experiment, where red dots represent CD11c$^+$ B cells, while the distribution of CD11c$^+$ cells among the 4 quadrants presented as percentages are means of 8 independent for tonsillar, and of 5 independent for blood-derived B cells. The effect of activation on the distribution of CD11c$^+$ B cells (mean +/- SD) was calculated from the results of 8 donors for tonsillar and of 5 donors for blood-derived B cells using two-way ANOVA with Sidak post-test (*=$p < 0.05$; **=$p < 0.01$; ***=$p < 0.001$).

4.2. Role of CR4 on BCR-activated tonsillar memory B cells

4.2.1. CR4 mediates adhesion of activated B cells to fibrinogen coated surface

The involvement of CR4 is well known in the adhesion of myeloid cells, where it was found to have a dominant role over CR3, even when expressed to a smaller extent (54). On B lymphocytes CR4 was also demonstrated to be involved in the adhesion of PMA stimulated B cells (1). However, it is important to point out, that PMA is a non-physiological stimulator of the cells having a strong effect on integrins themselves (110), thus the measured function of CR4 on PMA stimulated cells may not represent the physiological role mediated by CR4 on B cells.

For this, we set out to assess the adhesive capacity of B cells activated via the physiologically relevant BCR-stimulus. Since one of the major natural ligands for CR4 is fibrinogen, we assessed the adhesive capacity of BCR-activated cells to fibrinogen coated surface. We found, that blocking the function of CR4 with a CD11c specific antibody significantly decreased the adherence of B lymphocytes (**Figure 11**). Samples, where isotype control antibody treated cells were let to adhere to a surface without fibrinogen coat, served as negative control. In this case adherent cells could barely be detected; thus, our data clearly demonstrate that CR4 specifically mediates adhesion of activated human B cells to fibrinogen, the natural ligand of this β_2 integrin.
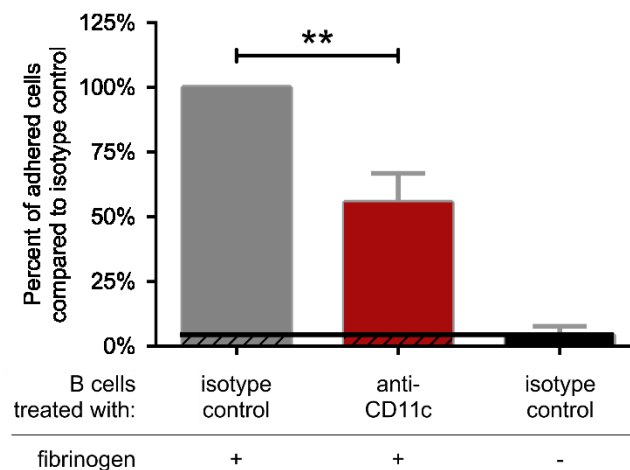
Figure 11. CR4 mediates adhesion of activated human B cells to fibrinogen. BCR-stimulated human tonsillar B cells were let to adhere to surfaces coated with fibrinogen and blocked by PLL-PEG (grey and red columns) or to surfaces without fibrinogen (black column). Adherence was performed in the presence of CD11c specific or isotype control antibody. The diagram summarizes the results (mean +/- SD) calculated from 4 independent experiments using one-way ANOVA with Tukey's post-test (*=p < 0.05; **=p < 0.01; ***=p < 0.001).

4.2.2. CR4 mediated adhesion enhances the proliferation of B cells

Cell adhesion is known to be involved in the proliferation of B lymphocytes, as shown e.g. in the case of adhesion to fibronectin via very late antigens VLA-4 and VLA-5 (122). Fibrinogen, which is the natural ligand of CR4 was found in high amounts on the surface of follicular dendritic cells (FDCs) in the dark zone of germinal centers (GCs) of human tonsils (123), where antigen-activated B cells proliferate extensively. The proliferating B cells are always found in close contact with FDCs (124), and integrin-ligand interactions between B cells and FDCs were found to play important roles during GC responses (125). These findings suggest that adhesion of activated B lymphocytes to fibrinogen via CR4 could also support this process. Lefevre et al. showed ealier, that fibrinogen enhances the proliferation and survival of the BCR-stimulated L3055 cell line, which represents a clonal population of centroblasts (123). This process however, has not been tested so far on primary B cells.

So, as we found that CR4 mediates the adhesion of human B lymphocytes to fibrinogen, we set out to investigate whether this process affects the proliferative capacity of the cells. We carried out the proliferation assay on fibrinogen coated surface where non-specific binding sites were blocked by PLL-PEG. As controls, we employed non-treated surface, where cells could adhere to any undefined structure, and PLL-PEG treated surface, where adhesion was hindered.

We found that the proliferative capacity of BCR-activated cells was significantly decreased on PLL-PEG, underlining the importance of adhesion for proliferation. On fibrinogen-coated surface however, the proliferative capacity of the BCR-activated lymphocytes was significantly increased, demonstrating the involvement of CR4 in this process (**Figure 12**).
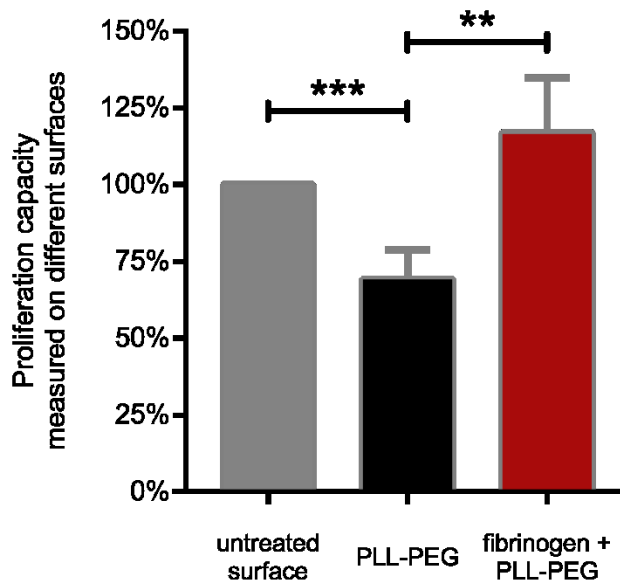
Figure 12. **B cell proliferation is significantly enhanced by CR4 mediated adhesion.**
The proliferation of BCR-activated cells was measured on non-treated (grey) PLL-PEG-
treated (black) or fibrinogen coated plus PLL-PEG treated (red) surface. Data are
normalized to the proliferation rate measured on non-treated surface (indicated as 100%,
grey column). The diagram summarizes results (mean +/- SD) calculated from 7
independent experiments using one-way ANOVA with Tukey's post-test (*=p < 0.05;
**=p < 0.01; ***=p < 0.001).

4.2.3. CR4 contributes to the migration of activated B cells

To reach germinal centers recirculating resting B cells migrate in the lymph nodes through
the network of FDCs. The CR4-ligand, fibrinogen was found on the surface of FDCs in the dark
zone of germinal centers (123). B cell access to the dark zone is dynamically regulated by the
expression of the C-X-C chemokine receptor 4 (CXCR4) (126), driving B lymphocytes towards
the chemoattractant SDF-1 (stromal cell-derived factor-1), produced by the reticular cells of the
dark zone (127). CD11c^{+} B cells are also known to differentiate into antibody-secreting cells
upon activation (6), which then home preferentially to the bone marrow (128), where SDF-1 is
also expressed (128,129).

Aiming to reveal, whether CR4 expressed by activated human B cells could participate
in this important function, we analysed the migration of BCR-activated B lymphocytes through
fibrinogen covered membranes towards SDF-1. We found, that blocking CR4 with a ligand

binding site specific antibody significantly decreases this function, as compared to the isotype control antibody treated cells (**Figure 13**). The measurement was performed in a transwell assay using fibrinogen coated and PLL-PEG blocked membranes. We used isotype control antibody treated cells as negative control, which were let to migrate through a PLL-PEG blocked membrane without the fibrinogen coat, and/or in the absence of the chemoattractant in the lower chamber (black columns on Figure 13).
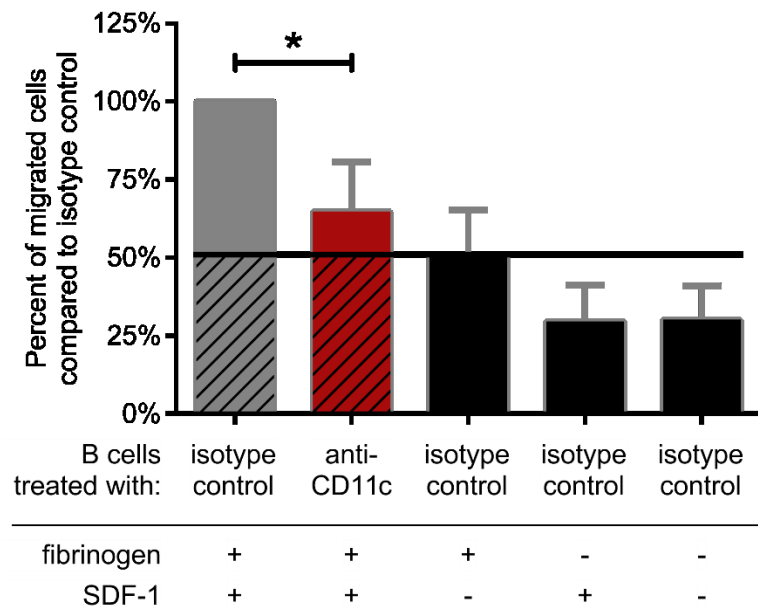

B cells treated with:	isotype control	anti-CD11c	isotype control	isotype control	isotype control
fibrinogen	+	+	+	-	-
SDF-1	+	+	-	+	-

Figure 13. Migration of BCR-stimulated cells through fibrinogen coated membrane towards SDF-1 is blocked by CD11c specific antibody. Cells were treated with CD11c-specific antibody (red), and the number of transmigrated cells were counted by flow cytometry. Results of 6 donors are shown as mean +/- SD, normalized to the control sample presented as 100% (grey). As negative control isotype control antibody treated cells were let to migrate through PLL-PEG blocked membrane without fibrinogen and/or in the absence of the chemoattractant SDF-1 (black columns) One-way ANOVA with Tukey's post-test was used to determine significant differences compared to control (*=$p < 0.05$; **=$p < 0.01$, ***=$p < 0.001$).

4.3. Integrin expression of malignant B cells

4.3.1. Expression and function of CR3 and CR4 on the B cell line BJAB

CR3 and CR4 are also known to be expressed in various B cell malignancies (7–14,112), where their function and contribution to the pathomechanism is still in question. To investigate the role of the receptors on malignant cells, a commonly applied experimental approach is the use of cell lines.

In our preliminary studies we tested whether the cells of the EBV-negative Burkitt-like lymphoma line BJAB could serve as a model for examining the role of CR3 or CR4 expressed by malignant human B cells. Flow cytometry measurements revealed that BJAB cells are positive for CD11c, but negative for CD11b (**Figure 14A**), similarly to the activated B cells of healthy donors (**Figure 6**). As CR4 was found to be involved in the adhesion of activated normal B cells (**Figure 11**), we set out to measure the adhesive capacity of BJAB cells to fibrinogen, the natural ligand of CR4. We found that blocking CR4 by a CD11c specific antibody significantly decreased the number of adherent cells compared to control (**Figure 14B**). Thus, CR4 is able to fulfil its function on malignant cells as well, and the Burkitt's lymphoma-derived BJAB cells can be a useful model for studying the role of CR4 on malignant human B cells.

However, it is important to point out, that cell line studies can only give information about the *possible* role of the receptors, but they do not prove that the receptors can *actually* carry out the found functions on primer cells. To reveal the actual role of these integrins on malignant cells investigation carried out using primary cells is inevitable. Thus, we decided to continue our studies on primary B cells isolated from the blood of chronic lymphocytic leukaemia patients.
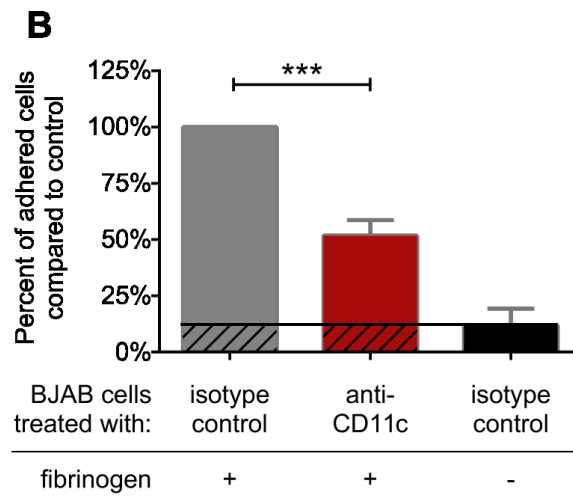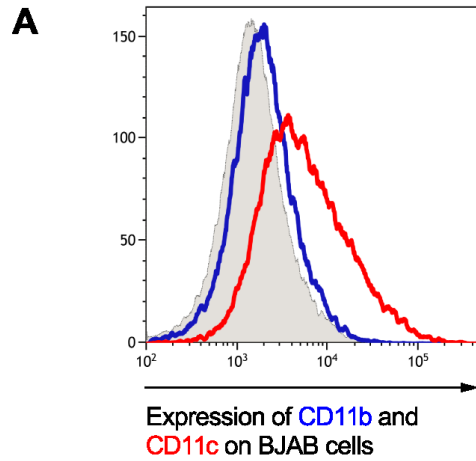
Figure 14. CR4 is expressed by the cell line BJAB and contributes to adherence. CR3 (blue) and CR4 (red) expression was measured by flow cytometry. Result of one representative experiment of 5 independent one is shown (**A**). BJAB cells were let to adhere to fibrinogen coated and PLL-PEG blocked surface in the presence of CD11c specific (red) or isotype control antibodies (grey). Results of 5 independent experiment are shown (mean +/- SD). One-way ANOVA with Tukey's post-test was used to assess significant differences compared to control (*=$p < 0.05$; **=$p < 0.01$; ***=$p < 0.001$) (**B**).

4.3.2. Expression of CR3 and CR4 on B cells of CLL patients

The anatomical distribution of the different B cell malignancies can be partially explained by the profile of the adhesion molecules expressed on the B cell surface. For this reason, it has a special importance that integrins and other cell adhesion molecules have been shown to have an important role in the motility and trafficking of cancerous B cells (111). Amongst others CR3 and CR4 have been also detected on the B cells of chronic lymphocytic leukaemia patients (7–14), where the level of their expression was found to correlate with the progression of the disease (7,8,10,11). However, the function of these receptors on CLL cells is still unexplained.

Here we selected eight CLL patients, whose B cells express at least one of these β_2 integrins. Blood derived B cells were cultured in the presence of IL-2 and anti-IgG/A/M F(ab')$_2$ antibody, to ensure survival of the malignant cells *in vitro*. CD11b and CD11c expression was measured by flow cytometry directly after isolation and after 3-day culture. We found, that although unstimulated CLL B cells already express CD11c, in most of the cases the expression of this β_2 integrin can be further stimulated by BCR-activation, similarly to that found on B cells of healthy donors (**Figure 6**). While all of the examined patients' B cells expressed CD11c at each time point, the expression of CD11b varied by patients and time (**Figure 15**).

	day 0			day 3		
	CD11b	CD11c	histogram	CD11b	CD11c	histogram
Patient 1	-	+		-	++	
Patient 2	-	++		-	+++	
Patient 3	-	+		+	+++	
Patient 4	++	++		++	+++	
Patient 5	+	++		++	+++	
Patient 6	-	+		-	+++	
Patient 7	-	++		+	++	
Patient 8	+	+		-	++	
Healthy	-	-		-	++	
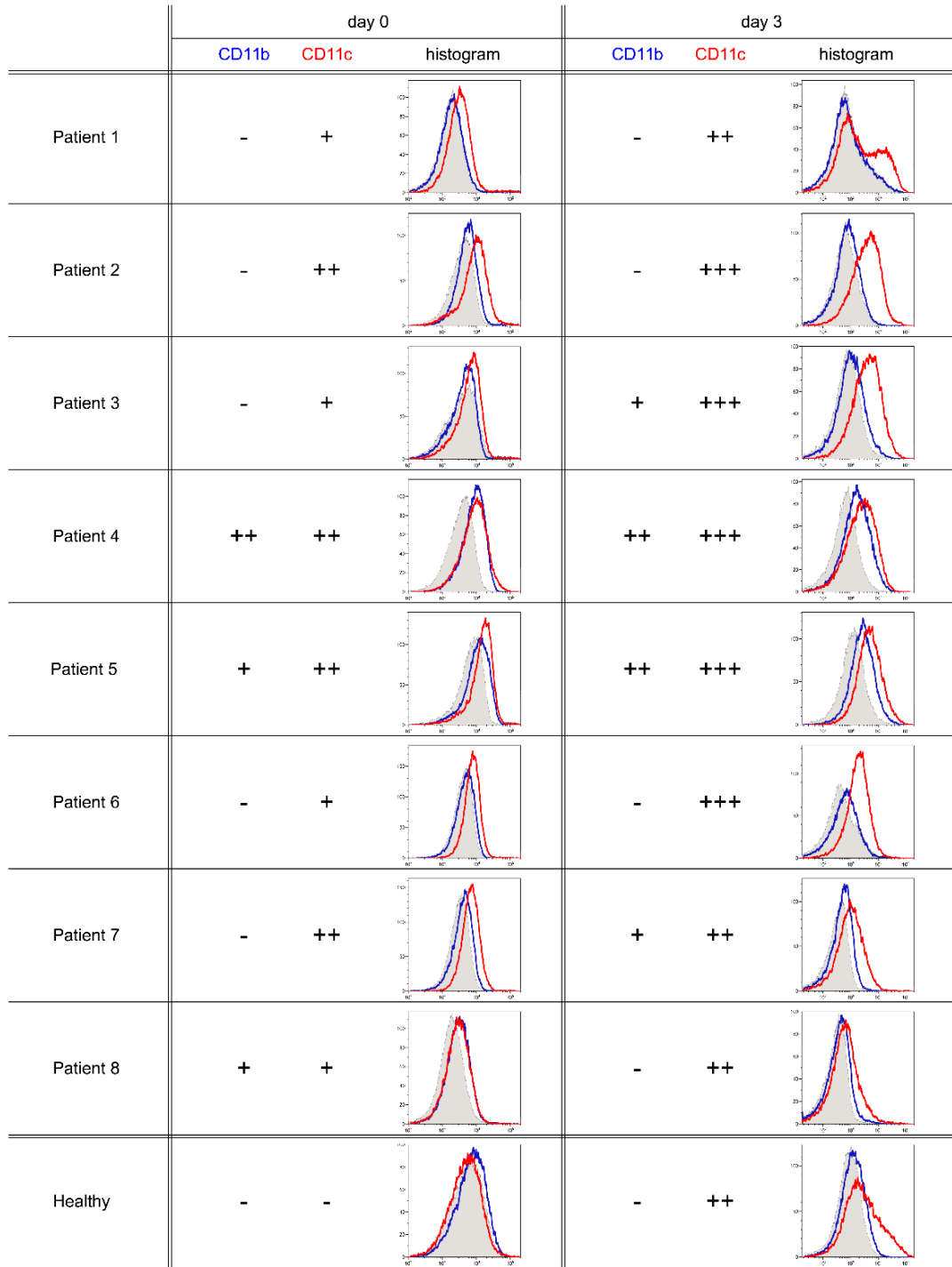
Figure 15. Expression of CD11b and CD11c on B cells of CLL patients directly after isolation and on the 3rd day of culture. CR3 (blue) and CR4 (red) expression was measured by flow cytometry on B cells of eight CLL patients directly after isolation and on the 3rd day of cell culture. As controls, B cells of healthy donors were investigated. Expression was ranked positive if relative mean fluorescence intensity (RMFI) was higher than 150%. Positivity was ranked by the number of + symbol as follows: RMFI <150% '-', 150-200% '+', 200-500% '++', >500% '+++').

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4.3.3. Expression of $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ on B cells of CLL patients

Using fibrinogen - the natural ligand of CR3 and CR4 - during the functional assays, we deemed important to test whether other fibrinogen-binding integrins ($\alpha_5\beta_1$ (CD49e/CD29), $\alpha_v\beta_3$ (CD51/CD61), or $\alpha_{IIb}\beta_3$ (CD41/CD61) (20,23)) could also appear on CLL B cells, as no systematic studies were done regarding their potential expression until now. Out of these receptors only $\alpha_5\beta_1$ (CD49e/CD29) was shown previously to be expressed on healthy or malignant human B cells (130–132), however, since ectopic gene activation was found to occur at a large scale in almost any type of tumour cells, we found it critical to analyse the expression of the other fibrinogen binding integrins as well.

We detected CD41a and CD49e on the B cells of Patient 8, while none of these integrins were expressed by the unstimulated B cells of healthy donors (**Table 4**). Moreover, both CD41a and CD49e expression increased after 3 days of BCR-stimulation in the case of CLL patients, resulting in CD41a$^+$ CLL B cells in four (Patient 2, 3, 4, 5) and CD49e$^+$ CLL B cells in six (Patient 2, 3, 4, 6, 7, 8) of the studied cases. In contrast, only CD49e was expressed by BCR-stimulated B cells of healthy donors. (BJAB cells were also tested, but none of these integrins were detected.)

	day 0			day 3		
	CD41a	**CD51**	**CD49e**	**CD41a**	**CD51**	**CD49e**
Patient 1	-	-	-	-	-	-
Patient 2	-	-	-	+	-	+++
Patient 3	-	-	-	+	-	+
Patient 4	-	-	-	++	-	++
Patient 5	-	-	-	+	-	-
Patient 6	-	-	-	-	-	++
Patient 7	-	-	-	-	-	+
Patient 8	+	-	+	-	-	+++
Healthy	-	-	-	-	-	+

Table 4. Expression of CD41a, CD51 and CD49e on freshly isolated CLL B cells and on the 3rd day of culture. Expression was measured by flow cytometry on freshly isolated B cells of CLL patients and after three days of culture. In the case of healthy donors, expression levels were calculated as mean of 4 independent experiments. Positivity was ranked by the number of + symbol as follows: RMFI <150% '-', 150-200% '+', 200-500% '++', >500% '+++'.

4.4. Role of CR3 and CR4 on B cells of CLL patients

4.4.1. Both CR3 and CR4 mediate adhesion of CLL B cells

Since CD11c was found to play a key role in the adhesion of activated B cells of healthy donors (**Figure 11**), as well as in the case of the Burkitt-like lymphoma cell line BJAB (**Figure 14B**), we set out to investigate whether CR4 has a similar function on CLL B cells. As these malignant cells display a homogeneous memory phenotype (115), we assumed, that CR4 may exert a similar function as on activated memory B cells of healthy donors. Moreover, both CR3 and CR4 were found previously to be involved in the spreading of CpG-activated CLL B cells on fibrinogen (**Figure 5**)(12), still their role in adhesion was not proven yet.

Since in some cases CLL B cells were found to express not only CR3 or CR4, but CD41a or CD49e as well, we also analysed the role of these integrins in the adhesion to fibrinogen. We found, that blocking the function of either CR3 (blue) or CR4 (red) with specific antibodies significantly decreased the adherence of CLL B cells compared to the control samples (grey) (**Figure 16**). Samples, where isotype control antibody treated cells were let to adhere to a surface without fibrinogen coat, served as negative control (black). As seen in Figure 16, CR4 contributes to the adhesion of CLL B cells to fibrinogen, similarly to that observed in the case of BJAB cell line (**Figure 14**) and activated tonsillar B cells (**Figure 11**). Moreover, we found that CR3, when expressed by CLL B cells, is also able to fulfil this function. Interestingly, while both CD41a and CD49e were previously shown to mediate adhesion to fibrinogen on platelets (118) and on endothelial cells (133), the effect of CD41a and CD49e specific antibodies was not significant in the adhesion of CLL B cells (**Figure 17**).
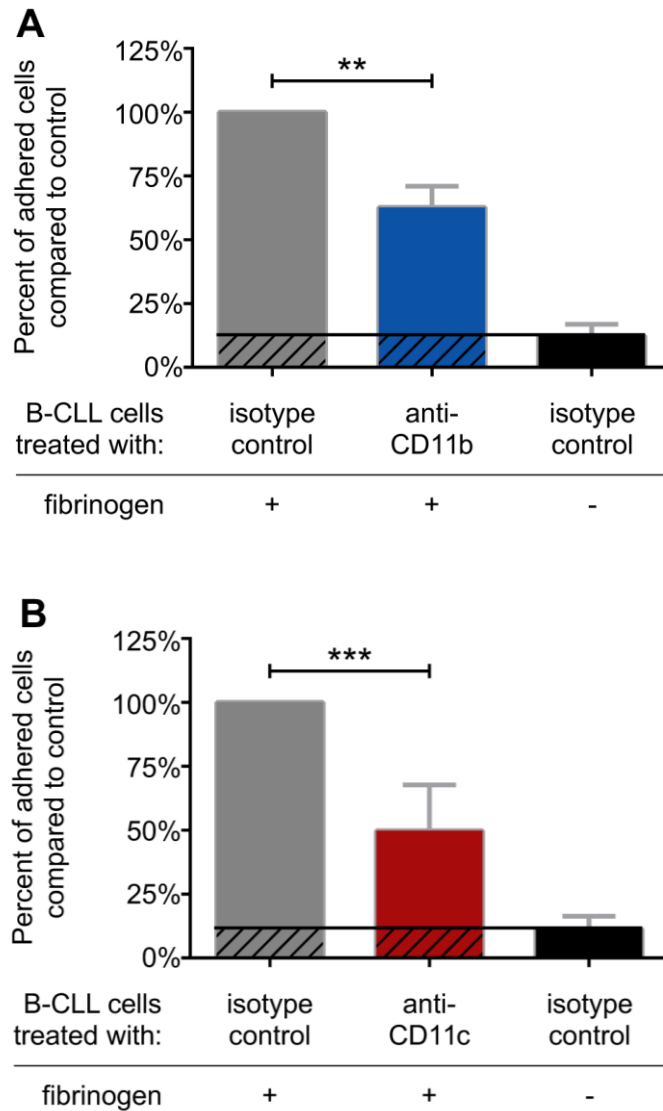
Figure 16. Both CD11b and CD11c specific antibodies inhibit the adhesion of CLL B cells to fibrinogen coated and PLL-PEG blocked surface. Cells were let to adhere to surfaces coated with fibrinogen and blocked by PLL-PEG or to surfaces without fibrinogen as negative control. Adherence was performed in the presence of CD11b- (**A**) or CD11c- specific antibodies (**B**). For CD11b, results of four patients (Patient 3, 4, 5, 7), for CD11c, results of eight patients are summarized as mean +/- SD, normalized to control presented as 100%. One-way ANOVA with Tukey's post-test was used to determine significant differences compared to control (*=$p < 0.05$; **=$p < 0.01$; ***=$p < 0.001$).
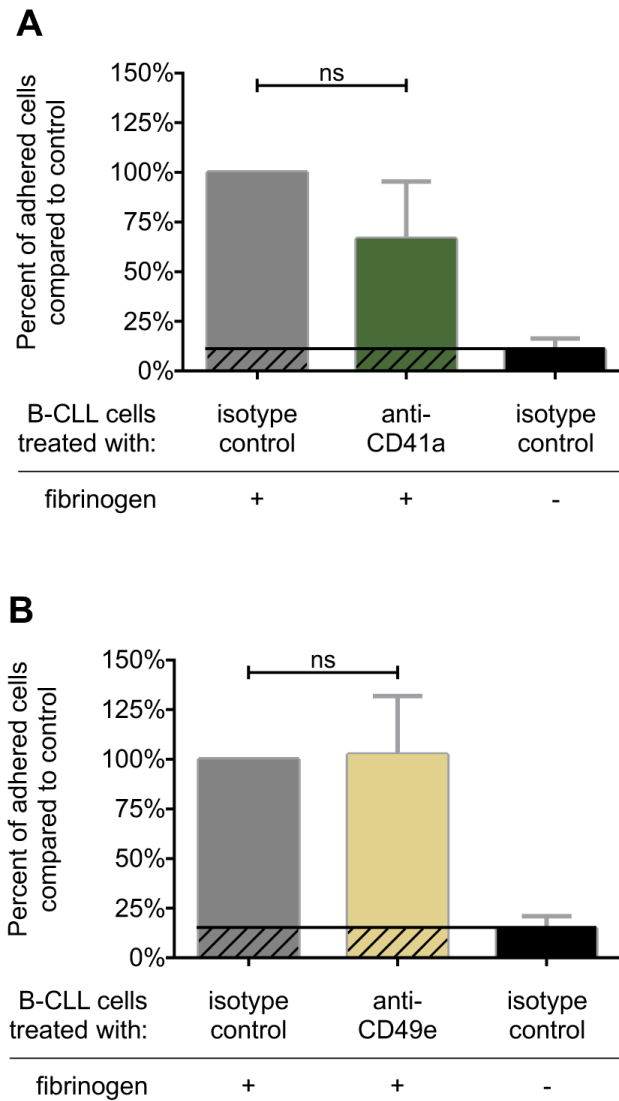
Figure 17. CD41a and CD49e specific antibodies have no significant effect on the adhesion of CLL B cells to fibrinogen coated and PLL-PEG blocked surface. Cells were let to adhere to surfaces coated with fibrinogen and blocked by PLL-PEG or to surfaces without fibrinogen as negative control. Adherence was performed in the presence of CD41a- (**A**) or CD49e- specific antibodies (**B**). For CD41a results of four patients (Patient 2, 3, 4, 5), for CD49e results of six patients (Patient 2, 3, 4, 6, 7, 8) are summarized as mean +/- SD, normalized to control presented as 100%. One-way ANOVA with Tukey's post-test was used to determine significant differences compared to control.

4.4.2. CR4 dominates over CR3 in the migration of CLL B cells towards SDF-1

Chronic lymphocytic leukaemia is characterized by the accumulation of clonal memory B cells within the blood, lymph nodes, spleen, bone marrow and other lymphatic tissues, where they crowd out healthy blood cells (114,115). The proliferating compartment of CLL exists in the bone marrow and lymph nodes, where the stromal microenvironment provides anti-apoptotic and pro-survival signals (116). Moreover, this stromal microenvironment can contribute to the chemoresistance as well (117). These characteristics of CLL pathomechanism explains, why every factor, that affects the dissemination of the malignant B cells, can potentially influence the outcome of the disease.

For this reason, we set out to analyse the involvement of CR3 and CR4 in the migration of CLL B cells. In the assays we used SDF-1 as chemoattractant, which directs the malignant cells to the bone marrow, and provides survival signal to them, and is thereby a key contributor to the pathomechanism of CLL (116,134). We found that inhibiting CR4 with a CD11c specific antibody resulted in a significant decrease in the number of migrated CLL B cells (**Figure 18B**), while the CD11b blocking antibody caused no significant decrease (**Figure 18A**). This suggests that the capacity of the two β_2 integrins is not identical, and CR4 dominates over CR3 in the migration of CLL B cells. We assessed the migration through fibrinogen-coated and PLL-PEG blocked transwell membrane. As negative control, we performed the assay in the absence of SDF-1 and/or using a transwell membrane which was not coated with fibrinogen, only blocked with PLL-PEG.
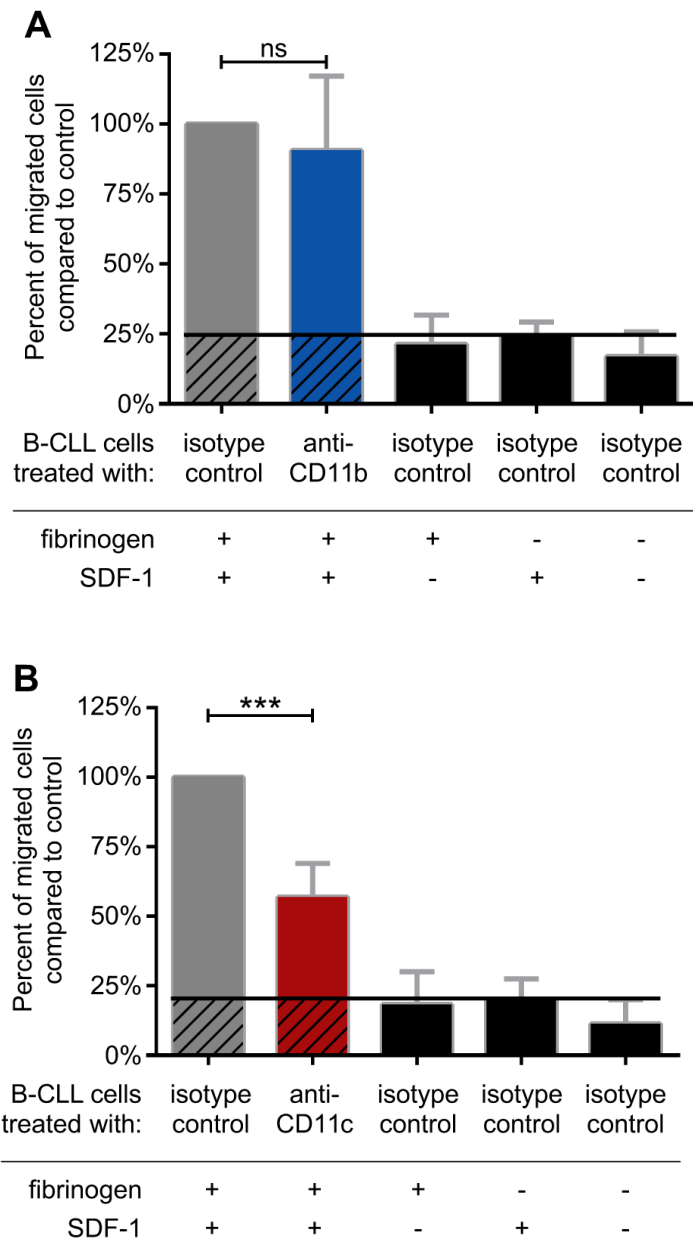
Figure 18. CD11c specific antibody blocks the migration of CLL B cells through fibrinogen coated and PLL-PEG blocked membrane towards SDF-1. CD11b- (**A**) or CD11c- (**B**) specific antibody was present throughout the assay, and the number of transmigrated cells were counted by flow cytometry. Results of four patients for CD11b (Patients 3, 4, 5, 7) and eight patients for CD11c are shown. Mean +/- SD is normalized to control presented as 100%. One-way ANOVA with Tukey's post-test was used to determine significant differences compared to control (*=p < 0.05; **=p < 0.01, ***=p < 0.001).

5. Discussion

CR3 and CR4 are complement receptors belonging to the family of β_2 integrins. Their role is well established in myeloid cell types, where they contribute to phagocytosis, adhesion and migration. Previously these receptors were commonly referred to have similar functions, however, recently we have suggested that they should rather be considered as "non-identical twins", due to their newly uncovered distinct roles in actin-linked functions of human phagocytes (27). Namely, our group demonstrated that CR4 has a dominant function in the adhesion to fibrinogen of human monocytes, dendritic cells and macrophages (54), while CR3 plays a more important role in the phagocytosis by the same cells (68).

The appearance and function of these complement receptors are known for long on myeloid cells, however, studies aiming to reveal their expression and role in human B lymphocytes have begun only recently. Moreover, the data produced by these recent studies are sometimes also inconsistent (reviewed in Erdei et al. 2019 and 2021 (27,135)). For example according to Ehrhardt and colleagues the expression of these β_2 integrins on human B cells is mutually exclusive (136), while Golinski et al. demonstrated that the expression of the mRNA for CD11b can be upregulated in CD11c^{+} B cells (6). The appearance of these receptors was demonstrated on certain subpopulation of B cells or after activation by different stimuli (1–6) as well as in various B cell malignancies such as chronic lymphocytic leukaemia (7–14). However, the role of CR3 and CR4 expressed by B cells has only scarcely been studied so far.

Thus, we planned and carried out a systematic study in order to clarify the expression and function of these receptors on primary human B cells under physiological conditions as well as in the case of a B cell malignancy. To this end, we characterized the expression of CR3 and CR4 on resting and activated B cells of healthy donors, and studied their role in adhesion, migration and proliferation. We also investigated CR3 and CR4 of B cells of chronic lymphocytic leukaemia patients.

5.1. Expression of CR3 and CR4 on B cells of healthy donors

In earlier studies several groups have shown that human B cells express CR3 although in largely varying ratio. Namely Muto et al. detected CD11b on 20% of human peripheral B cells (93), while Rothstein et al. identified a small subset of B1 cells to be CR3^{+} (2) and Kawai and colleagues found one-third of B lymphocytes to express CD11b (5). CR4 was also detected on

around 20% of blood B lymphocytes by Wormsley et al. (3). On the other hand according to Rubtsov and colleagues only low numbers of CD11c^{+} B lymphocytes can be found in the blood of healthy donors, however, these cells are expanded in older women with autoimmune disease (4). In line with these findings, Golinski et al. demonstrated recently, that the ratio of CD11c^{+} B cells in blood correlates with the age of the healthy donors, varying between 3 to 55 percentages (6). To darken counsel, others detected neither of these receptors on resting B lymphocytes (1,12). At the same time however, it was also found, that the expression of CD11c is upregulated after activation. Namely, PMA treatment (1) as well as activation via the B cell receptor enhanced the expression of CR4, while TLR9 or TLR7 mediated activation did not result in CD11c expression (6).

In our systematic study first we measured the expression of CR3 and CR4 on the surface of resting B cells as well as on activated lymphocytes by flow cytometry. Investigating the effect of activation on integrin expression we deemed important to choose physiologically relevant stimuli, thus we activated B cells via the BCR and TLR9. Since peripheral lymphoid organs - such as tonsils – are the primary sites for B cell activation, moreover they contain a wider range of various B cell subsets than blood, we assessed the expression of CD11b and CD11c both on tonsillar and blood B cells.

We found that while resting B cells express neither of these β_2 integrins, BCR-mediated activation resulted in CD11c expression in around 26% of tonsillar B lymphocytes, while CD11b could not be detected neither at mRNA, nor at protein level. After stimulation via TLR9 approximately 11% of B cells became positive for CD11c, and this ratio was elevated up to 21% when the cells were simultaneously triggered via the BCR and TLR9 (**Figure 6**). Our results showing that BCR-stimulation leads to the upregulation of CR4 are in line with the recent finding of Golinski et al., however, in their study they found that this expression could not be induced by the ligation of TLR7 and TLR9 (6). At the same time, Rubtsov et al. demonstrated in mice, that the accumulation of CD11c^{+} B cells is driven by TLR7 (4). While these data seems to suggest a differential regulation of CR4 expression in human and mouse B lymphocytes, the contradictory results could also be ascribed to the different methods applied. Regarding the effect of TRL9 mediated activation our results also seem to contradict with the finding of Golinski et al. However, it is important to point out, that Golinski et al. tested how sorted CD11c^{-} cells react to activation, and whether they turn on receptor expression after TLR stimulation, which did not occur (6). In contrast to this, we stimulated the whole B cell pool, and found an increase in the number of CD11c^{+} B cells. Therefore, it is possible, that in our

experimental conditions TLR9-stimulation induced the expansion of CR4$^+$ B cells and not the transition of CD11c$^-$ cells to CD11c$^+$ ones.

As activation through BCR was clearly the strongest physiological trigger to induce CR4 expression, we focused on BCR-stimulated B lymphocytes. To explore the mechanism and kinetics of BCR-activation induced expression of CD11c, we analysed this process at the mRNA as well as the protein level simultaneously for three days following activation. This experiment was motivated by the fact that even if the kinetics of protein-expression is determined mostly by transcription and translation, β_2 integrins can be transferred rapidly to the surface from intracellular sources following activation, as described in the case of other cell types (93). Our measurements proved clearly that in the case of B cells, CR4 appears on the surface of activated B cells as the result of *de novo* protein synthesis (**Figure 7**).

Regarding the density of CR4 we revealed that on average 9500 CD11c molecules are present on the cell surface on the third day of BCR-activation (**Figure 8**). This is a higher number than that detected on monocytes (54) or neutrophil granulocytes (27), thus it was reasonable to assume that this scale of expression is high enough to fulfil certain functions of B cells.

It is important to point out, that the activity of the integrins is conformationally regulated, and they are able to bind their ligands and exert various functions only in their extended, active conformation. Therefore, we analysed the conformation of β_2 integrins and found that they are expressed on BCR-activated CD11c$^+$ B cells in their functionally active state (**Figure 9**).

We also studied the phenotype of CD11c$^+$ B lymphocytes, and found that most of them belong to different memory B cell subsets (**Figure 10**), which is in line with earlier studies (4,6). The finding, that the majority of CD11c$^+$ B cells are memory B cells can also explain the lower number of CD11c$^+$ cells among the TLR9 stimulated B lymphocytes, as TLR9 agonists trigger an intense differentiation into plasmablasts and plasma cells, thus the ratio of memory B cells is lower in this case (137). However, since CD11c appeared to some extent on each subpopulation, it may have a role in each step of B cell development (6).

As the previous studies focused mostly on blood-derived B cells, we set out to characterize the phenotype of tonsillar B cells as well. We found, that similarly to blood B cells, the vast majority of tonsillar CD11c$^+$ B lymphocytes can also be characterized as switched memory cells. Lower amount of CD11c$^+$ B lymphocytes have been detected among unswitched memory B cells and only a few CD11c$^+$ cells were found in the double negative or naive

populations (**Figure 10A**). Thus, despite the fact that tonsils and blood contain different B cell populations, the distribution of CD11c$^+$ cells among non-stimulated B cells of blood and tonsils is very similar (**Figure 10B**). Nevertheless, BCR-activation resulted in significantly higher number of switched and significantly lower number of unswitched memory B cells among tonsillar B cells, a phenomenon which could not be detected in the case of blood-derived B lymphocytes (**Figure 10A**). This means that the development of CD11c$^+$ B cells after BCR-activation occurs in parallel with Ig class switching in tonsils.

5.2. Function of CR4 on B cells of healthy donors

Taken together, our data prove that CR4 is expressed in functionally active conformation by activated memory B cells at a level that is high enough to exert important functions. Based on these results we aimed to reveal, which functions can be mediated by CR4 of activated B cells. As fibrinogen is one of the major natural ligands of this β$_2$ integrin, we assumed that CR4 might contribute to the adhesion and migration of B lymphocytes at anatomical sites where fibrinogen is present. Lefevre et al. found high amounts of fibrinogen on the surface of follicular dendritic cells in the dark zone of GCs of human tonsils (123), where both the generation and re-activation of memory B cells occur. In their paper the authors raised that "further studies will be required to identify the discrete B cell population present in GC, which responds to fibrinogen". The results of our studies clearly show that CR4 expressed by activated B cells mediates adherence to fibrinogen (**Figure 11**), thus they represent the fibrinogen-responsive B cell population referred to by Lefevre.

Fibrinogen was also shown to trigger the proliferation and survival of the BCR-activated L3055 cells, which represent a clonal population of centroblasts (123). Here we analysed the effect of fibrinogen on the activated B cells of healthy donors and found that binding to fibrinogen also increased the BCR-induced proliferation of the primary tonsillar B cells (**Figure 12**). This fibrinogen mediated enhancement of BCR-triggered proliferation very likely occurs *in vivo* within human tonsils when antigen-activated B lymphocytes form GCs in close contact with the FDC network (**Figure 19**). Follicular dendritic cells maintain immunological memory by antigen presentation and also by supporting B cell survival and proliferation via additional mechanisms (138). According to our results, activated B cells likely use CR4 to establish a close contact with the fibrinogen-covered FDCs, which contact promotes the binding of antigens by B cells and also provides additional proliferation and survival signals. Moreover,

since adhesion to fibrinogen enhances the proliferation of BCR-activated B cells, we also suggest, that the CR4 mediated adhesion itself could be one of the supporting signals B cells receive from the fibrinogen-covered FDCs in the dark zone. This effect might be similar to that found previously in the case of other integrins. Namely, LFA-1 and VLA-4 were also found to mediate the adhesion of tonsillar B cells to FDCs (139), moreover, LFA-1 also helped the synapse formation of B lymphocytes, and thus lowered the threshold of B cell activation (140). As a feedback, activation via BCR was found to promote both the LFA-1 (141) and VLA-4 mediated adhesion (142).

It is crucial to pay attention to the fact, that while the majority of FDCs are located in the light zone, the fibrinogen-covered FDCs were detected only in the dark zone of GCs (123). The dark zone serves as the main site for proliferation and somatic hypermutation of B lymphocytes (143), where they are recruited with the help of CXCR4 (126). This chemokine receptor directs B cells towards the chemoattractant SDF-1, which is produced by the reticular cells of the dark zone (127). To find out whether CR4 could also have a role in this process, we analysed the contribution of CD11c to the SDF-1 mediated migration of activated B cells. We found that CR4 mediates the migration of activated B cells towards SDF-1 as well (**Figure 13**) proving the important role of CR4 in GC reactions (**Figure 19**). Furthermore, it is also possible, that CD11c$^+$ B cells, as they differentiate into antibody-secreting cells upon activation (6), use CR4 during their homing into the bone marrow, where SDF-1 is also expressed (129).
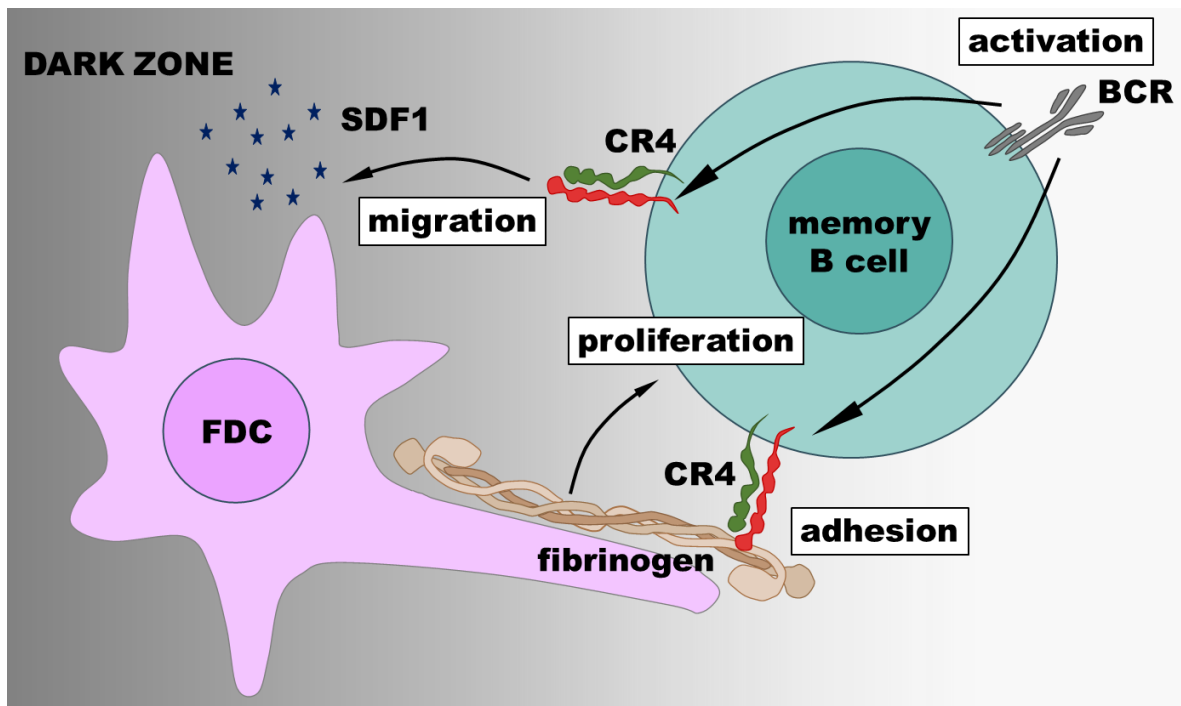
Figure 19. Activated human memory B lymphocytes use CR4 for adhesion, migration, and proliferation. CR4 is synthesized by memory B cells after activation via the BCR. The CD11c^{+} B cells use CR4 to mediate adhesion to fibrinogen, which is present in high amounts on FDCs in the dark zone of GCs of human tonsils. Binding to fibrinogen enhances the BCR-induced proliferation of B lymphocytes. This phenomenon is very likely to occur *in vivo* within human tonsils when B cells are activated by antigen and form GCs in close contact with the FDC network. We suggest, that adhesion to fibrinogen by CR4 itself could be one of the supporting signals B cells receive from FDCs. We also found that CR4 is involved in the migration of activated B cells towards SDF-1 which finding gives a strong support to the important role of CR4 in the GC reactions, as B cell access to the dark zone, and thereby to the fibrinogen-covered FDCs, is regulated by this chemoattractant.

5.3. Expression of CR3 and CR4 on B cells of CLL patients

Malignant B cells are known to overexpress numerous members of the integrin family, which have special importance in the disease pathomechanism as well, since the profile of the expressed adhesion molecules can shape the anatomical distribution of the B cell lymphomas (111). β_2 integrins were found to be expressed by diffuse large B cell lymphoma, mantle-cell lymphoma, Hodgkin's lymphoma, CLL and Burkitt's lymphoma cells (111), from which hairy cell leukaemia (9), Hodgkin's lymphoma (112) and CLL (7,8,10) have been demonstrated to express CR3 and/or CR4 as well. At the same time however, the role of the expressed complement receptors has not been clarified on these malignant B cells yet. After we found, that CR4 is expressed on activated B cells of healthy donors and is involved in various functions, we aimed to clarify whether CR4 retains its capacities in B cell malignancies as well, and whether it may even serve as an active driver of the disease.

First we studied the CD11c^{+} Burkitt's lymphoma cell line BJAB, as a representative of malignant B cells. Analysing the involvement of CD11c in the adhesion of BJAB cells to fibrinogen we found that it contributes significantly to this function of these cells as well (**Figure 14B**), similarly to that found in the case of activated B cells of healthy donors (**Figure 11**). This finding proves that CR4 is expressed not only as a marker of activation, but can act as a functionally active molecule by malignant B cells, too. However, as detailed previously, we are aware that experiments carried out using cell lines cannot replace experiments done with primary cells.

This is why we decided to perform experiments using cells isolated from the blood of CLL patients. These malignant cells are typically characterized as monoclonal, CD5^{+}CD19^{+}CD23^{+} dysfunctional B cells, which accumulate in the blood, secondary lymphoid tissues and bone marrow, where they crowd out healthy blood cells (114). CR3 and CR4 expression was detected on the malignant cells in several patients, however, the prevalence of CD11b or CD11c positive cases varied highly in the available studies. Namely this rate of incidence was found to be 21% (10), 26% (7), 28% (8), 40% (11), 49% (9) or even 90% (14) for CR4 expression, while the ratio of CD11b positive cases varies from 20% (8) to 66% (10). In the present study we selected eight patients, whose B cells were positive for at least one of these β_2 integrins. Out of them, all of the analysed patients' B cells were positive for CR4, while only few patients carried CR3^{+} cells (**Figure 15**).

Numerous studies demonstrated that CLL cells are in their activated state *in vivo*, hence chronic lymphocytic leukaemia is referred to as a disease of activated monoclonal B cells (144). When CLL cells are isolated from their *in vivo* environment, they are devoid of the activating factors, thus without *in vitro* stimulation their response would not mirror their *in vivo* behavior. For this reason we found it important to culture the isolated CLL cells in the presence of IL-2 and anti-IgG/A/M, and carry out the functional assays with activated cells, as these stimulators are naturally present and needed for the functions of CLL cells (145,146). Thus we also measured the expression of CR3 and CR4 on CLL B cells after culturing them in the presence of IL-2 and anti-IgG/A/M. We found that the activating environment facilitated the expression of these integrins as well, as it boosted the expression of CR3 in two cases and further elevated the expression of CR4 in seven cases out of the studied eight (**Figure 15**).

Using fibrinogen - the natural ligand of CR3 and CR4 - during the functional assays, we deemed important to test whether other fibrinogen-binding integrins ($\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_{IIb}\beta_3$) could also appear on CLL B cells, as no systematic studies were done regarding their potential expression until now. One of those, $\alpha_5\beta_1$ (also known as CD49e/CD29 or VLA-5) is known as the "classical" fibronectin receptor, but have been shown to bind fibrinogen as well (133,147). It was shown to have a regulated expression during human B cell differentiation and activation (130,131), with expression at a very early stage and then again after activation (130). On malignant B cells it's expression was shown to correlate with the capacity of malignant B lymphocyte dissemination (132). $\alpha_v\beta_3$ (also known as CD51/CD61) is a receptor for vitronectin, and is expressed in the highest number on osteoclasts (148). $\alpha_{IIb}\beta_3$ (CD41/CD61) is the major integral plasma protein of platelets, but it can be found on megakaryocytes, mast cells and basophils as well (149).

While out of these receptors only $\alpha_5\beta_1$ was shown previously to be expressed on healthy or malignant human B cells, as ectopic gene activation was found to occur at a large scale in almost any type of tumour cells, we found it critical to analyse the expression of all the mentioned fibrinogen-binding integrins on CLL B cells. While unstimulated B cells of healthy donors did not express any of these molecules, we detected CD41a and CD49e on freshly isolated B cells of Patient 8. Moreover, both CD41a and CD49e expression increased after three days of BCR-stimulus (**Table 4**).

5.4. Function of CR3 and CR4 on B cells of CLL patients

While the appearance of both CR3 and CR4 correlates with the incidence of bone marrow infiltration (7) and is listed as an unfavourable prognostic factor in chronic lymphocytic leukaemia (7,8,10,11), the exact mechanism of how they contribute to the pathomechanism of CLL was still in question. At the same time, CLL B cells were characterized as having a homogeneous phenotype typical of memory B cells (115), moreover, they are known to be in their activated state (144). Therefore we assumed, that CR4 may exert a similar function as on activated memory B cells of healthy donors. Analysing the involvement of CR3 and CR4 to the adhesion to fibrinogen – which was mediated by CR4 in case of activated memory B cells of healthy donors (**Figure 11**) – we found, that both of these β_2 integrins can contribute to this process (**Figure 16**). Interestingly, while both CD41a and CD49e was found to bind fibrinogen on other cell types earlier, neither of them contributed significantly to the adhesion of CLL B cells in our study (**Figure 17**).

CLL B cells are known to proliferate in the bone marrow and lymph nodes (150), where they gain anti-apoptotic and survival signals from the stromal microenvironment (116). Additionally, stromal cells are also shown to provide chemoresistance (117), and it was found that while most therapeutic agents are capable to eradicate leukemic cells from the blood, residual disease remains in the bone marrow and lymph nodes (116). The recruitment of CLL B cells towards these stromal cells is driven by the stromal cell-derived factor-1 (SDF-1). This chemoattractant was also shown to exert a direct survival-promoting effect on the leukemic cells (134).

Based on these reports we found it important to investigate whether the expressed CR3 and CR4 could contribute to the migration of CLL B cells towards SDF-1. We found that CR4 has a significant role in this function of CLL cells (**Figure 18B**), while CR3 might only have a minor role during migration (**Figure 18A**). Since adhesion is the first step and prerequisite of migration, we have not tested the possible contribution of CD41a or CD49e to CLL migration.

Importantly, the finding that CR4 mediates the migration of CLL B cells towards SDF-1 makes clear, how the expression of CD11c associates with bone marrow infiltration. Moreover, as stromal cells as well as SDF-1 play a key role in the pathomechanism of CLL, this function of CR4 explains how its appearance could be an unfavorable prognostic factor in CLL.

Taken together, we revealed a mechanism, how CR3 and CR4 might contribute to the progression of chronic lymphocytic leukaemia (**Figure 20**). Importantly, overcoming adhesion-mediated resistance is essential in developing new therapies, as it was also proven in the case of another integrin, VLA-4. Natalizumab, the blocking anti-VLA-4 antibody, has been demonstrated to decrease B lymphocyte adherence to stroma and thereby partially control stromal protection towards rituximab and cytotoxic drugs (151). We suggest that therapeutic antibodies to CR4 might have similar effects and along with anti-CR3 antibodies, they could hopefully even complete the partial effect of natalizumab and other treatments.
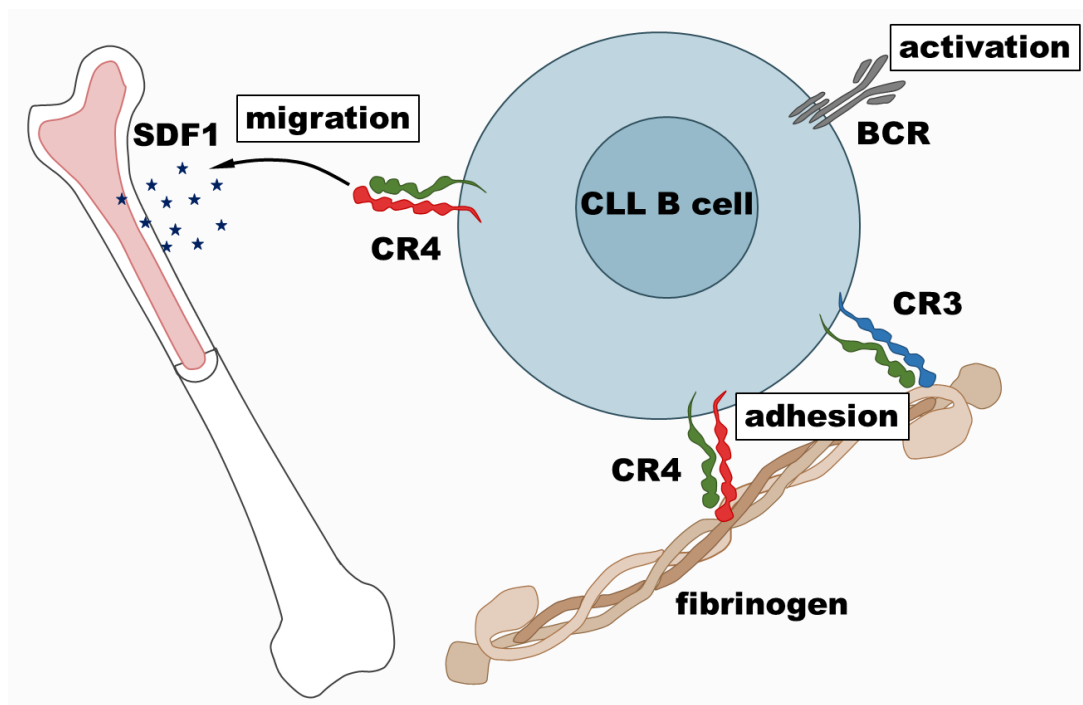

Figure 20. Both CR3 and CR4 contributes to the adhesion while CR4 has a dominant role in migration towards SDF-1 in the case of BCR-activated B cells of CLL patients. B cells of CLL patients can express both CR3 and CR4 and the presence of either of them correlates with the pattern of bone marrow infiltration, thereby being an unfavourable prognostic factor. We showed that both receptors contribute to the adhesion of CLL B cells, while CR4 has a dominant role in the migration towards SDF-1. This chemoattractant has a key role in the pathomechanism of CLL, driving migration towards the stromal microenvironment of the bone marrow where they proliferate and gain resistance to a wide variety of treatments. This role of the stromal microenvironment together with the contribution of CR4 to the migration towards SDF-1 explains why the expression of CD11c associates with bone marrow infiltration.

Summary

The expression of CR3 and CR4 was demonstrated in certain populations and on activated B cells of healthy donors, as well as in various B cell malignancies, such as in CLL. Still, their exact role in human B cells have only scarcely been studied so far. Thus, we characterized the expression and function of CR3 and CR4 on B cells of healthy donors and CLL patients.

We found that while neither of these receptors appear on resting B cells, after activation via the BCR and TLR9 they express CR4, but not CR3. We proved that CD11c appearing on the B cell surface are newly synthesized and their level of expression is high enough to exert important functions. The vast majority of CD11c$^+$ cells belong to different memory B cell subsets, yet, CD11c was detected in each subpopulation, suggesting a role for CR4 in each step of B cell development. We also found that the increase of CD11c expressing cells among the BCR-activated tonsillar lymphocytes occurs in parallel with Ig class switching.

Regarding its function, we found, that CR4 mediates the adhesion of B cells to fibrinogen, which is present in high amounts on the surface of FDCs in the dark zone of GCs, where both the generation and re-activation of memory B cells occur. We also proved that binding to fibrinogen increases the BCR-induced proliferation of tonsillar B cells; a process, which very likely occurs *in vivo* within human tonsils, when antigen-activated B lymphocytes form GCs in close contact with the FDC network. According to our results, activated B cells use CR4 to establish a close contact with the fibrinogen-covered FDCs, moreover, the CR4 mediated adhesion provides a supporting signal for the lymphocytes. We also found that CR4 is involved in the migration towards SDF-1. This finding further supports the important role of CR4 in the GC reactions, as B cell access to the dark zone is regulated by this chemoattractant.

In the case of CLL B cells both CR3 and CR4 were found to be expressed. While the presence of either CR3 or CR4 is known to correlate with the pattern of bone marrow infiltration, their exact role on malignant B cells was still in question. We have shown that both CR3 and CR4 contribute to the adhesion to fibrinogen. Regarding migration towards SDF-1 however, CR4 proved to exert a dominant role. SDF-1 has a key role in the pathomechanism of CLL, driving cancerous cells towards the stromal microenvironment where they proliferate and gain resistance to a wide variety of treatments. Thus, the contribution of CR4 to the migration towards SDF-1 explains why the expression of CD11c is associated with bone marrow infiltration. Our results are of particular interest, as overcoming adhesion-mediated resistance is important in developing new therapies against chronic lymphocytic leukaemia.

Összefoglalás

Bár a CR3 és a CR4 jelenlétét kimutatták bizonyos B sejt populációkon, aktivációt követően, illetve leukémiás B sejteken is, emberi B sejteken betöltött szerepük máig tisztázatlan volt. Ezért vizsgáltuk e két receptor megjelenését és szerepét egészséges donorok és CLL betegek B sejtjein. Azt találtuk, hogy míg egészséges, nyugvó B sejteken egyik receptor sem fejeződik ki, a sejtek aktiválása a CR4 megjelenését váltja ki. A CD11c molekulák új fehérjeszintézis eredményeképp jelennek meg, és kellő mennyiségben fejeződnek ki ahhoz, hogy érdemi funkciót töltsenek be. A CD11c$^+$ B sejtek többsége memória fenotípusú, bár valamennyi CD11c$^+$ sejt minden B sejt alpopulációban megtalálható, vagyis a CR4 a B sejt-fejlődés minden lépésében szerepet játszhat. Ugyanakkor a CD11c megjelenése jellemzően izotípusváltással párhuzamosan zajlik le a BCR-aktivált mandula eredetű B sejtek esetében.

Az aktivált B sejteken megjelenő CR4 részt vesz a fibrinogénhez történő kitapadásában. Ez a fehérje nagy mennyiségben található meg azon follikuláris dendritikus sejtek (FDC-k) felszínén, melyek a csíracentrumok sötét zónájában helyezkednek el, ahol a memória B sejtek kialakulása, valamint újraaktiválódása is lezajlik. Bizonyítottuk, hogy a fibrinogénhez való kitapadás a BCR-aktivált B sejtek osztódását is segíti. Ez a folyamat nagy valószínűséggel *in vivo* is lezajlik az emberi nyirokcsomókban, amikor az antigén-aktivált B sejtek csíraközpontokat hoznak létre szoros kapcsolatban az FDC-kkel. Eredményeink alapján ezt a kapcsolatot az aktivált B sejtek a CR4 segítségével alakítják ki, sőt, maga a CR4 közvetített kitapadás is osztódási szignált jelent számukra. Kimutattuk, hogy a CR4 szerepet játszik az SDF-1 felé irányuló migrációban is. Ez szintén a CR4 csíraközpont reakciókban betöltött fontos szerepére utal, hiszen ez a kemoattraktáns szabályozza a B sejtek sötét zónába jutását.

CLL betegek B sejtjein mind a két receptor megjelenik, megjelenésük pedig összefüggést mutat a csontvelői infiltrációval. Ennek ellenére a betegség lefolyásában betöltött pontos szerepük nem ismert. Kimutattuk, hogy mindkét receptor részt vesz a CLL sejtek fibrinogénhez való kitapadásában, ugyanakkor az SDF-1 irányú migrációban a CR4 játszik főszerepet. Az SDF-1 kulcsfontosságú a betegség lefolyásában, mivel a leukémiás sejteket a csontvelő mikrokörnyezetébe vonzza, ahol azok osztódnak és rezisztenssé válnak a kemoterápiával szemben. Így a CR4 SDF-1 irányú migrációban betöltött szerepe megmagyarázza, miért függ össze a CD11c kifejeződése a csontvelői infiltráció gyakoriságával és a rossz prognózissal. Ezek az eredmények különösen jelentősek annak fényében, hogy az új, CLL elleni terápiák fejlesztésében kulcsfontosságú az adhézió-közvetített rezisztencia kiküszöbölése.

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List of publications

Publications connected to the thesis

1. <u>Nagy-Baló Z</u>, Kiss R, Menge A, Bödör C, Bajtay Z, Erdei A.
   Activated Human Memory B Lymphocytes Use CR4 (CD11c/CD18) for Adhesion,
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2. <u>Nagy-Baló Z</u>, Kiss R, Demeter J, Bödör C, Bajtay Z, Erdei A.
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   adhesion while CR4 has a dominant role in migration towards SDF-1.
   PLoS One. 2021;16(7 July):1–14.

Further publications

3. Lukácsi S, <u>Nagy-Baló Z</u>, Erdei A, Sándor N, Bajtay Z.
   The role of CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in complement-mediated
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